August, 1953 *Price 35s. net* Vol. 9, No. 1

THE JOURNAL OF GENERAL MICROBIOLOGY

QR
1
J64

EDITED FOR

THE SOCIETY FOR GENERAL MICROBIOLOGY

Editors

B. C. J. G. KNIGHT A. F. B. STANDFAST

Associate Editors

W. B. BRIERLEY
S. T. COWAN
T. GIBSON
N. G. HEATLEY
J. H. HUMPHREY
A. T. R. MATTICK
A. A. MILES
P. MITCHELL
R. H. NIMMO-SMITH
K. M. SMITH
A. W. STABLEFORTH
D. D. WOODS

CAMBRIDGE UNIVERSITY PRESS
LONDON: BENTLEY HOUSE, N.W.1
NEW YORK: 32 EAST 57TH STREET, 22

THE
JOURNAL OF GENERAL MICROBIOLOGY

is conducted by the

SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology was founded in order to promote the advancement of microbiology by providing a common meeting ground for those working in all the various specialized fields including, for example, medical, veterinary, agricultural and economic microbiology. The Society is concerned with the more fundamental aspects of the study of bacteria, viruses, micro-fungi, protozoa and microscopic algae in their various biological activities, its members being united by the common aim of developing scientific knowledge concerning micro-organisms.

The annual subscription, which entitles members to receive the *Journal of General Microbiology*, is 35*s*. and is payable on 25 March each year.

OFFICERS OF THE SOCIETY

President

H. J. Bunker, M.A., Research Department, Barclay Perkins & Co., Park St., London, S.E. 1

Treasurer

R. Lovell, D.Sc. M.R.C.V.S., Vine Cottage, Streatley-on-Thames, Berks.

General Secretary

J. G. Davis, D.Sc., 52 London Road, Reading, Berks.

Meetings Secretary

E. F. Gale, Sc.D., M.R.C. Unit for Chemical Microbiology,
Department of Biochemistry, Cambridge

All correspondence concerning payment of subscriptions and any other financial matters should be addressed to the Treasurer. All correspondence concerning meetings should be sent to the Meetings Secretary and all correspondence on other matters, including applications for membership and changes of address, should be sent to the General Secretary.

All communications concerning the purchase of the Journal, including the purchase of back numbers by members of the Society, should be addressed to the Cambridge University Press.

Members are particularly asked to write to the appropriate officer on matters concerning their membership privileges.

PURCHASE OF JOURNAL BY NON-MEMBERS

For non-members of the Society the subscription for the *Journal of General Microbiology*, volumes 8 and 9, is £4. 10*s*. 0*d*. each, post free ($15.00 in U.S.A.). These volumes will be completed in three parts, the price of these separate parts is £1. 15*s*. 0*d*. each, plus the cost of postage.

The price of back volumes can be had on application to the

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE
200 EUSTON ROAD, LONDON, N.W.1

or through any bookseller.

Enquiries from the U.S.A. should be addressed to the Cambridge University Press, American Branch, 32 East 57th Street, New York, 22.

Applications for advertising space should be sent to the Cambridge University Press.

THE JOURNAL OF GENERAL MICROBIOLOGY

EDITED FOR
THE SOCIETY FOR GENERAL MICROBIOLOGY

Editors
B. C. J. G. KNIGHT A. F. B. STANDFAST

Associate Editors
W. B. BRIERLEY
S. T. COWAN
R. DAVIES
T. GIBSON
N. G. HEATLEY
J. H. HUMPHREY
A. T. R. MATTICK
A. A. MILES
P. MITCHELL
R. H. NIMMO-SMITH
K. M. SMITH
A. W. STABLEFORTH
D. D. WOODS
K. ZINNEMANN

VOLUME 9, 1953

CAMBRIDGE
AT THE UNIVERSITY PRESS
1953

Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)
and published by the Cambridge University Press
London: Bentley House, N.W. 1
American Branch: New York
Agents for Canada, India, and Pakistan: Macmillan

Kernforschungsanlage
Jülich
Zentralbibliothek

Pa 2770/ZB (PC | Prof. Kaul)

Contents

No. 1 (*issued* 26 *August* 1953)

PAGE

The Establishment of Serologically Identifiable Strains of *Rhizobium trifolii* in Field Soils in competition with the Native Microflora. By Margaret P. Read. With a Note by G. V. Dyke 1

The Fermentation of Malt Extract by an Osmophilic Yeast. By Mary P. English. With a Note by J. T. Gunner and Constance Rainbow 15

A Probable Growth Cycle in *Bacillus megaterium*. By F. J. Bergersen 26

Radiation-induced Instabilities in Streptomyces. By H. B. Newcombe 30

Interrelationships between Amino acids in the Growth of Coliform Organisms. By D. Rowley 37

The Cultivation and Metabolism of Oligotrich Protozoa from the Sheep's Rumen. By Brenda Sugden 44

The Release of Soluble Constituents from Washed Cells of *Pseudomonas aeruginosa* by the action of Polymyxin. By B. A. Newton . 54

The Vi Type-determining Phages carried by *Salmonella typhi*. By E. S. Anderson and A. Felix 65

The Utilization of Nitrate Nitrogen by the Azotobacter. By Margaret Green and P. W. Wilson 89

The Relation of Bacteriophage Pattern to some Biological Properties of Staphylococci. By E. Levy, Joan E. Rippon and R. E. O. Williams 97

Micromethod for the Methyl Red Test. By S. T. Cowan . . . 101

The Identification of Strains of *Streptococcus pyogenes* of Types 5, 11, 12, 27 and 44 by the Precipitin Test for the T-Antigen. By Sibely J. McLean 110

Influenza Virus Multiplication in the Chick Chorio-allantoic Membrane. By F. Fulton and A. Isaacs 119

Interference in the Chick Chorion. By A. Isaacs and F. Fulton . 132

Antigenic Relationships of Swine Influenza Virus. By Annette E. H. Gompels 140

Mycobacterium tuberculosis var. *muris*. By A. Q. Wells . . . 149

Proceedings of the Society for General Microbiology

COMMUNICATIONS

Effect of Aeration on the Citric Acid Metabolism of *Aerobacter aerogenes*. By S. Dagley and E. A. Dawes i

PAGE

Some Carbohydrate-fermenting Organisms isolated from the Rumen of the Sheep. By SHEILA M. WILSON i

The Diaminodicarboxylic Acids of Actinomycetales. By ELIZABETH WORK ii

Acetate, Amino-acid Synthesis and the Tricarboxylic Acid Cycle in *Escherichia coli.* By K. McQUILLEN iii

Cystathionine in relation to Methionine Synthesis by *Bacterium coli.* By S. WIJESUNDERA and D. D. WOODS iii

Some Interrelationships between Nicotinic Acid, Pyridoxin and Purines in the Growth of a Strain of *Saccharomyces cerevisiae.* By J. W. MOULDER and D. D. WOODS iv

Characteristic Mutants of Species in the *Aspergillus niger* Group and their Behaviour in Mixed Culture. By J. L. YUILL . . . v

Nutritional Conditions which increase the Ability of *Aspergillus niger* to form α-Ketoglutarate from Citrate. By A. N. HALL and T. K. WALKER vi

A Preliminary Report of the Application of Electrokinetic Methods to the Study of Bacterial Adaptation. By A. M. JAMES and P. J. BARRY vi

Apparent Adaptation to *in vivo* Conditions in *Pasteurella pestis.* By T. W. BURROWS vii

The Adaptive Oxidation of L-Histidine by certain Bacteria. By H. TRISTRAM vii

Auto-adaptation: a New Phenomenon observed in a Bacterial Population. By BARBARA E. WRIGHT viii

The Fermentation of Raffinose by *Saccharomyces fragilis* Jorgensen. By R. DAVIES ix

Variation in a small Colony Strain of Staphylococcus. By C. H. SMITH ix

Inhibition by Amino acids of the Growth of Coliform Organisms. By D. ROWLEY x

Genetic Analysis of Valine Inhibition in the K12 Strain of *Escherichia coli.* By A. MANTEN and D. ROWLEY x

The Selection of Amino acid Inhibited Mutants of *Escherichia coli.* By R. C. CLOWES and D. ROWLEY x

SYMPOSIUM ON ADAPTATION IN MICRO-ORGANISMS

Adaptation, Evolutionary and Physiological; or Darwinism among the Micro-organisms. By R. Y. STANIER xi

Observations on Bacterial Adaptation. By A. C. R. Dean and Sir CYRIL HINSHELWOOD xi

PAGE

The Nature of Variations affecting Bacterial Adaptability. By A. W. Ravin xi

Stages in Enzyme Adaptation. By M. R. Pollock xi

The Nature of the Precursor in the Induced Synthesis of Enzymes. By S. Spiegelman and H. O. Halvorsen xi

Specific Induction and Inhibition of Enzyme Biosynthesis. By M. Cohn and J. Monod xi

A Specific Relation between Enzymatic Adaptation and Cytoplasmic Mutation. By P. P. Slonimski xii

The Effect of Temperature on Enzymic Adaptation, Growth and Drug Resistance. By R. Knox xii

The Development of Drug Resistance in Micro-organisms. By E. P. Abraham xii

Antibiotic-resistant Staphylococcal Variants. By Mary Barber . xii

The Ecology of Tubercle Bacilli Resistant to Streptomycin and Isoniazid. By D. A. Mitchison xii

Influence of Bacteriophage on Bacterial Variation and Evolution. By L. F. Hewitt xii

Adaptations in Paramecium. By G. H. Beale xii

Environmental and other Aspects of Adaptation. By L. F. L. Clegg and S. E. Jacobs xii

Ecological Adaptations in Fungi. By W. Brown and R. K. S. Wood xii

No. 2 (*issued* 8 *October* 1953)

The Integration of Research on the Nutrition and Metabolism of Micro-organisms. By D. D. Woods 151

The Influence of Cultural Conditions on Polysaccharide Production by *Aerobacter aerogenes*. By J. P. Duguid and J. F. Wilkinson . 174

A Strain of *Lactobacillus bifidus* which requires Strepogenin. By H. Gyllenberg, Marjatta Rossander and P. Roine 190

The Study of Cell Rupture in *Staphylococcus aureus*. By P. D. Cooper 199

A Method for Direct Viable Counts of the Facultatively Anaerobic Microflora in the Rumen of a Sheep maintained on a Hay Diet. By P. J. Heald, N. Krogh, S. O. Mann, J. Christine Appleby, Frances M. Masson and A. E. Oxford 207

The Effect of Fatty Acids on the Growth, Metabolism and Morphology of the Anaerobic Cocci. By P. Wildy and R. Hare 216

Genetic Analysis of Valine Inhibition in the K 12 Strain of *Bacterium coli*. By A. Manten and D. Rowley 226

PAGE

The Classification of Lactobacilli by means of Physiological Tests. By Mary Briggs 234

The Precipitating Antigens of Friedländer's Bacillus. By M. J. Pickett and V. J. Cabelli 249

Paths of Phosphate Transfer in *Micrococcus pyogenes;* Phosphate Turnover in Nucleic Acids and other Fractions. By P. Mitchell and Jennifer M. Moyle 257

Transport of Phosphate across the Surface of *Micrococcus pyogenes*: Nature of the Cell 'Inorganic Phosphate'. By P. Mitchell . 273

Electron Microscopy of a Staphylococcal Bacteriophage. By J. L. Farrant and Phyllis M. Rountree 288

Micromanipulation on an Agar Surface for the Isolation and Cultivation of Single Organisms. By K. I. Johnstone 293

Heterotrophy in Diatoms. By Joyce C. Lewin 305

Antibiotic Production by the Microfungi of Acid Heath Soils. By E. G. Jefferys, P. W. Brian, H. G. Hemming and D. Lowe . . 314

The Induction of Lysogenesis in *Salmonella typhimurium.* By W. R. Parry and Joyce Edwards 342

Growth of Streptococci in a Glucose Phenolphthalein Broth. By Patricia H. Clarke 350

No. 3 (*issued* 9 *December* 1953)

Cytological Changes induced in *Bacterium coli* by Chloramphenicol. By F. J. Bergersen 353

The Influence of the Host on Competition amongst Clover Root nodule Bacteria. By J. M. Vincent and L. M. Waters 357

Nutritional Requirements of Acetic Acid Bacteria. By C. Rainbow and G. W. Mitson 371

In vivo Observations on the Ciliate Protozoa inhabiting the Large Intestine of the Horse. By Katherine M. G. Adam 376

The Oxidation of Lard Oil by *Penicillium chrysogenum.* By G. N. Rolinson and M. Lumb 385

The Distribution of α,ϵ-Diaminopimelic Acid among various Microorganisms. By Elizabeth Work and D. L. Dewey. With a Note by R. L. M. Synge 394

Transduction of Flagellar Characters in Salmonella. By B. A. D. Stocker, N. D. Zinder and J. Lederberg. 410

The Isolation from Lemon Juice of a Growth Factor of Steroid Nature required for the Growth of a Strain of *Paramecium aurelia.* By R. L. Conner, W. J. van Wagtendonk and C. A. Miller . 434

PAGE

On the Nutrition of *Desulphovibrio desulphuricans*: a Correction. By J. R. Postgate 440

Factors affecting the Requirement of *Tetrahymena pyriformis* (*geleii*) for Folic Acid. By Virginia C. Dewey and G. W. Kidder . . . 445

The Absorption of Polymyxin E by Bacteria and Bacterial Cell Walls and its Bactericidal Action. By A. V. Few and J. H. Schulman 454

Some Effects of Sucrose and Phosphorus in increasing the Multiplication of Tobacco Mosaic Virus in detached Tobacco Leaves. By B. Kassanis 467

Some Factors influencing the Survival of *Bacterium coli* on Freeze-drying. By B. R. Record and R. Taylor 475

Growth Requirements of some Thermophilic and Mesophilic Bacilli. By H. Baker, H. Sobotka and S. H. Hutner 485

The Induction in a *Saccharomyces* sp. of the Gene-mutation controlling Utilization of Galactose by Exposure to Galactose. By C. C. Lindegren and D. D. Pittman 494

Cell Structure and the Enzymic Lysis of Bacteria. By M. R. J. Salton 512

The Acquired Resistance of *Staphylococcus aureus* to Bacteriophage. By E. J. L. Lowbury and A. M. Hood 524

Some Effects of 2-Deaminopteroylglutamic Acid upon Bacterial Growth. By R. H. Nimmo-Smith and D. J. Brown . . . 536

No. 3, Volume 8 of the Journal of General Microbiology was issued on 12th June 1953.

READ, M. P. (1953). *J. gen. Microbiol.* 9, 1–14.

The Establishment of Serologically Identifiable Strains of *Rhizobium trifolii* in Field Soils in competition with the Native Microflora

BY MARGARET P. READ

Department of Soil Microbiology, Rothamsted Experimental Station,
Harpenden, Hertfordshire

With a Note by G. V. DYKE on the
Statistical Examination of the Establishment Percentages

SUMMARY: Field trials were made at thirteen centres in varied localities to test whether a strain of clover *Rhizobium* used as a seed inoculum in the field could establish itself in the crop in competition with the native strains already present in the soil. Each experiment comprised four sets of plots, one uninoculated and the other three each sown with seed inoculated with a different strain of *Rhizobium*.

The difficulty in identifying a strain re-isolated from a nodule was met by using as inocula strains whose antigenic composition made them readily identifiable by agglutination tests. From each plot, twenty-five nodules were selected, and isolates from these tested against selected antisera. This method of identification enabled the percentage of nodules produced by each inoculant strain to be ascertained. Strains differed in their ability to establish themselves in the field: a suitable strain gave rise to 50% or more of the nodules. In sand culture competition between pairs of inoculant strains was not related to their ability to establish themselves in the field, but each strain whose establishment was superior also showed competitive dominance on at least one date of sampling.

The existence of ineffective strains of Rhizobium *in nature*

Ineffective strains of *Rhizobium*, incapable of supporting the nitrogen requirements of the leguminous plant by nitrogen fixation, seem to be prevalent among clover nodule bacteria, although they have been isolated also from soybean, pea, lupin and lucerne. A survey of the distribution of ineffective strains of *Rh. trifolii* in Great Britain has shown them to occur especially in poor hill pastures; plants from these areas are often pale and stunted and their roots bear a large number of small white nodules. Re-seeding is likely to fail if the new plants become infected with indigenous ineffective bacteria. Inoculation of the legume seed is a means of introducing effective strains, but there are various hazards that these bacteria must overcome before their establishment in the soil and in the clover can be assured.

The problem of inoculation

The literature contains many reports of the success of legume seed inoculation where the crop has not been grown before and appropriate root-nodule bacteria are known to be absent. When it is desired to replace a native population of ineffective strains with a relatively small inoculum of a beneficial strain, it is essential that this inoculum be able to withstand inter-strain competition. In the field, rhizobia invade the plant in the presence of a wide variety of organisms composing the indigenous flora of the soil and rhizosphere. The time lag between inoculation and invasion will be critical for eventual establishment of the inoculum, for competition with native strains may cause its suppression.

Nicol & Thornton (1941) found large differences between the rate of appearance and the final nodule numbers produced by various strains in pure culture, particularly with clover. When two strains were applied simultaneously to the roots, the final number of nodules due to each strain was not always related to its infectivity. Competition between the two strains in the rhizosphere could result in almost complete suppression of one strain. Dominance in competition seemed to be related to the initial rate of multiplication but not to the ability to fix nitrogen. In their experiments, the two competing strains were supplied in approximately equal numbers. Where seed inoculation is used for a crop such as clover, there is probably a great disparity between the numbers of introduced bacteria and those of the clover rhizobia already present in the soil. Wilson (1931) found that soils with a pH value suited to clover may be expected to contain some thousands of clover rhizobia/g. A culture of *Rh. trifolii* as used for seed inoculation contains some $2{\cdot}8 \times 10^7$ viable bacteria/ml. (Thornton, 1929), giving a spread of several hundred organisms on each seed-coat. Thus the number of bacteria introduced into the soil by the seed may be very small in an environment where rhizobia of the appropriate cross-inoculation group may be expected in normal numbers already. It was therefore necessary to ascertain whether such an inoculum was able to withstand competition from a large population of native nodule bacteria and to establish itself in the roots of the crop.

Serological identification of the inoculant strain

It is well established that the root nodule bacteria present a serologically heterogeneous group, but the relative scarcity of known antigenic structures enables certain selected strains to be identified by serological tests. Kleczkowski & Thornton (1944) found that strains agglutinating with 'O' antisera against *Rh. trifolii* P11 and 205 were of rare occurrence in British soils. This suggested the possibility of using for field inoculation strains containing one or other of these antigens as 'tracers' to distinguish nodules produced by the inoculum from those produced by native strains. This method was tested by Thornton & Kleczkowska (1950) at four different centres; the results showed that the serological method of nodule identification was practicable. They also indicated that the two strains used as inocula differed in their ability to establish themselves in competition with the local strains, although the number of nodule isolates tested in this preliminary trial was too small to be conclusive.

Objects of the present work

The experimental work to be described was undertaken to gain information concerning field establishment of effective strains of clover nodule bacteria, and in particular to discover:

(1) How large a percentage of nodules in an inoculated plot was produced by the inoculum.

(2) To what extent this percentage varied with the particular strain used as inoculum.

(3) How far the percentages produced by different strains varied with the local conditions.

FIELD EXPERIMENTS

Localities. In the spring of 1947, five field experiments were carried out at different localities. Four of these were carried on until the spring of 1948, when a further eight trials were also laid down. Details of the localities of these thirteen experiments are as follows:

1947, group A

Bradninch, Devon. Red sandstone, pH 6·9, 300 ft. O.D.
Middle Cator, Dartmoor, Devon. Black river sand, pH 6·2, 700 ft.
Harnhill, Glos. Limestone, pH 7·3, 300 ft.
Woburn, Beds. Sandy loam, pH 6·5, 300 ft.
Welshpool, Mont. Medium loam, pH 6·5, 300 ft.

1948, group B

Hoddesdon, Herts. Clay with gravel, pH 6·2, 100 ft.
Ingatestone, Essex. Heavy clay, pH 6·1, 200 ft.
Skipton, Yorks. Sandy lime over limestone, pH 6·5, 1000 ft.
Aberystwyth, Caernarv. Sandy loam, pH 6·9, 500 ft.
Cockle Park, Northumberland. Peaty loam, pH 5·2, 1000 ft.
Aberfoyle, Perth. Peaty loam, pH 5·4, 1000 ft.
Tornaveen I, Aberdeen. Medium sandy loam over granite, pH 6·7, 800 ft.
Tornaveen II, Aberdeen. Loam over granite, pH 6·5, 800 ft. (farm adjacent to Tornaveen I).

Plan and technique of field trials. Each experiment was carried out in a field which had not been sown to clover for several years, to avoid the risk of 'clover sickness'. A strip of sixteen randomized plots was laid down, each 5 × 3 yd. with a path 1 yd. wide between each to eliminate any carry-over of infection. Four plots were sown with uninoculated seed and four replicates with each of three effective strains of *Rh. trifolii.* Late-flowering Montgomeryshire red clover, *Trifolium pratense* Linn., was used throughout, and the inoculum was always supplied at sowing. The control plots became nodulated with native strains present in the soil, and an antigenic survey was necessary to distinguish them from strains used as inoculum. All plots were adequately fertilized with lime, phosphate and potash. It was hoped that infection of control plots with experimental strains would be avoided by seeding uninoculated plots first of all, and in keeping to the paths, to prevent infection from one plot to another. This was successful and cross-infection was very rare.

Strains used as inocula. The following strains of *Rh. trifolii* were selected:

(1) Clover F (GB) derived from Blandford, Dorset, England.

(2) 205 (US) obtained from Wisconsin Agricultural Experiment Station, U.S.A.

(3) 49 (GB) derived from Thame, Oxfordshire, England.

(4) 38 (GB) derived from Crickhowell, Brecknock, Wales.

(5) Pentyr 4 (GB) derived from Pentyr, near Cegin River, Caernarvon, Wales.

(6) 46 (AU) obtained from University of Sydney, Australia.

Some confusion exists owing to the same numbers having been applied to different strains in different countries. To avoid this confusion in the present paper, the letters (GB) specify strains isolated from British soils, and (US) and (AU) those isolated from soils of U.S.A. and Australia respectively. For simplicity these letters have been inserted here only in the description of the strains used and in Table 1. The strains used as inocula in 1947 were Clover F, 49 and 205; those in 1948 were Clover F, 38, Pentyr 4, and in one trial 46. The strains were originally isolated from effectively nodulated clover plants and had maintained their effectivity in pure culture for several years. Their antigenic constitution had remained constant, and furthermore they possessed distinguishing antigens (Kleczkowski & Thornton, 1944). It was hoped that the naturally occurring nodule bacteria would not possess these antigens.

Isolation of bacteria from nodules. After 2–3 months, when nitrogen fixation was under way, twenty-five plants were taken at random from each plot; a healthy nodule was selected from each plant, care being taken to include nodules of all sizes in this sampling. No inoculant strain could be recognized by the type of nodules it produced on plants in the field. It was thought preferable in this type of investigation to make isolations from one nodule selected from a large range of plants, rather than to sample more heavily from fewer plants, although Hughes & Vincent (1942) observed the occurrence of different serological types on the same plant. Each nodule was immersed for 3 min. in 80 % ethanol followed by 3 min. in 0·2 % mercuric chloride, and washed in sterile distilled water until the washings no longer contained chloride. The nodule was crushed in water to free the organisms from the central tissue, and the resulting suspension was streaked on agar of the following composition: mannitol, 1·0 %; K_2HPO_4, 0·5 %; $MgSO_4.7H_2O$, 0·02 %; NaCl, 0·02 %; $FeCl_3$, 0·001 %; aqueous yeast extract 10 %; agar 1·5 %. This medium gave discrete colonies in 3–4 days at 25°, after which a single colony was picked off and plated twice more. After the third successive plating on yeast mannitol agar, the cultures were examined for the presence of *Agrobacterium radiobacter* by the tests outlined by Hofer (1941); the cultures were not regarded as pure until this procedure was completed.

Serological testing of the isolates

Preparation of antisera. Suspensions for immunizing rabbits were prepared by mixing 2-day and 5-day yeast mannitol agar cultures to ensure an adequate cell content and a high proportion of actively motile bacteria (Vincent, 1941). One ml. of a heavy filtered suspension was injected intravenously every 5 days for 4 weeks, and the animal bled out when a tested sample had a titre against the somatic antigen of at least 1/3200. Although the somatic titre remained fairly constant in antisera stored at 4° over a considerable period, the flagellar titre progressively declined to 1/200 or less. Vincent (1941) and Kleczkowski & Thornton (1944) emphasized the need for ensuring the presence of motile bacteria, and all suspensions used throughout this work were checked for motility.

Agglutination test. As specific H and O antisera were not prepared for any strains, the agglutination test was carried out by the methods used by Kleczkowski & Thornton (1944). Isolates from the control plots were always tested first, and their somatic and flagellar reactions obtained separately, because in some strains the rapid appearance of O may mask an H reaction. With the inoculated plots, however, it was known that the inoculant strains searched for gave clear-cut H and O reactions, and it was thought safe to use untreated antiserum and to distinguish them by the very characteristic types of precipitate and the interval of their appearance. When control isolates did not give reactions identical with those of the introduced strains, it was assumed that the native microflora did not contain *Rh. trifolii* identical with the inoculum. In two trials some strains from the uninoculated plots gave reactions liable to be confused with those of the inoculant strains. In these, H and O reactions were obtained separately for all isolates, and inoculant strains were then clearly recognizable.

In the 1947 series, final dilutions of antiserum from 1/200 to 1/1600 were used, but in the 1948 series, tests were made only at 1/200; experience showed that many native rhizobia were of poor antigenicity. All isolates were tested against the test antisera, even where antisera shared a reaction, in order to obtain as much information as possible about the distribution of antigens in the soil population. Reactions were compared with a control tube of bacteria plus saline, and with those given by the homologous antigen. Atypical reactions were encountered sometimes, mainly as sparse floating floccules or rather mucoid precipitates, but the seven test strains gave clear-cut positive reactions with their homologous antisera. Occasionally an isolate reacted with all antisera (control negative) which may perhaps indicate the presence of a group antigen.

Validity of the technique. Altogether some 1700 isolates from uninoculated plots were tested. Of these only eight gave a combination of reactions identical with any of the inoculant strains. The figures show that the methods used were adequate to enable these strains to be identified in the plots to which they were added. For more precise serological analysis, further refinements such as the use of specific H and O antisera would have been desirable; owing to the necessity for using the minimum number of animals at the time this work was carried out, combined antisera had to be prepared.

Choice of antisera. Selection was based on the work of Kleczkowski & Thornton (1944), and in addition antisera which would give information of value to the geographical survey of clover strains in Great Britain. The strains and their corresponding antisera were as follows: (1) P11 (GB), antiserum against an effective strain from pea; (2) Clover F (GB), antiserum against strain Cl. F; (3) R 7A (GB), antiserum against strain R7A derived from clover near Aberdeen; (4) 49 (GB), antiserum against strain 49; (5) 46 (AU), antiserum against effective strain 46 used by Hughes & Vincent (1942) in agglutination studies of strains isolated in New South Wales; (6) 205 (US), antiserum against strain 205; (7) Orkney (GB) (included only in 1948 tests), strain derived from clover in the Orkney Islands. Thus antisera used throughout were prepared from strains isolated from widely scattered areas.

The H and O reactions to the seven antisera given by strains of *Rh. trifolii* used in this work are shown separately in Table 1, where titres are given as the reciprocal of the final dilution. Strains Clover F and 49 cross-react strongly, although they are not serologically identical. Their similarity did not, however, invalidate positive reactions given from plots inoculated with them since strains identical with either were absent from the uninoculated plots.

Table 1. *The agglutination titres of all the experimental strains of* Rhizobium trifolii *with all antisera*

The numbers are the reciprocals of the dilutions

	Antisera						
Antigens	P11 (GB)	Cl. F (GB)	49 (GB)	46 (AU)	205 (US)	Orkney (GB)	R7A (GB)
	Titre of reaction with corresponding antiserum						
Flagellar							
P11 (GB)	1600	800	400	—	—	—	—
Clover F (GB)	800	1600	800	—	—	—	—
49 (GB)	400	800	1600	—	—	—	—
46 (AU)	—	—	—	800	—	400	—
205 (US)	—	—	—	—	1600	—	—
38 (GB)	—	—	—	—	—	—	—
Pentyr 4 (GB)	—	—	—	—	—	—	—
Orkney (GB)	—	—	—	—	—	1600	—
R7A (GB)	—	—	—	—	—	—	800
Somatic							
P11 (GB)	3200	1600	800	—	—	—	—
Clover F (GB)	800	3200	800	—	—	—	50
49 (GB)	400	1600	3200	—	—	—	—
46 (AU)	—	—	—	3200	—	—	—
205 (US)	—	—	—	—	3200	—	—
38 (GB)	—	—	—	—	1600	800	—
Pentyr 4 (GB)	400	800	400	—	1600	—	—
Orkney (GB)	—	—	—	—	—	3200	—
R7A (GB)	—	—	—	—	—	—	3200

Establishment of inocula in the field

The results of a typical experiment are set out in Table 2, while the mean percentages of nodules due to each inoculum at the centres in the year of sowing are collected in Tables 3 and 4; this is referred to later as 'establishment'.

In every experiment it was possible, with one or other of the inocula, to obtain an establishment of over 30 %, and in all but two of the experiments, of over 50 %. There were large differences in the establishment achieved by different strains in the same trial. In the 1947 series (Table 3), the Welshpool experiment on land that had been a garden, showed no significant differences between strains, but at the four remaining localities, Clover F gave establishment ranging from 52 to 64 %, strain 49 from 31 to 41 % and strain 205 from 17 to 30 %. Thus the establishment obtained with each of these strains was characteristic of that strain and uniform over the four localities, independently of very diverse soil conditions.

Table 2. *Typical experiment showing the percentage nodulation due to each of three strains of* Rhizobium trifolii *at the Bradninch site*, 1947

Inoculum	Plot	Number tested	Number positive	Percentage positive	Mean percentage positive
Clover F	A1	25	16	64·0	63·2
	A2	23	13	56·5	
	A3	23	17	73·9	
	A4	24	14	58·3	
205	B1	24	6	25·0	30·1
	B2	24	8	33·3	
	B3	22	6	27·2	
	B4	23	8	34·7	
49	C1	24	9	37·5	40·0
	C2	22	11	50·0	
	C3	22	9	40·9	
	C4	22	7	31·8	
Uninoculated control	U1	21	None	—	—
	U2	21		—	—
	U3	22		—	—
	U4	24		—	—

Table 3. *Percentage of nodules containing the inoculant strains for the group A localities, with relevant standard errors (corresponding values in angular units shown in brackets)*

	Plots inoculated with strain				Critical difference (5 % level)
Group A localities	Clover F	205	49	S.E. (degrees)	(degrees)
1947					
Bradninch	63 (52·7)	31 (33·2)	40 (39·2)	±2·07	6·6
Middle Cator	53 (46·4)	17 (24·7)	31 (33·8)	±4·60	14·7
Harnhill	64 (53·2)	17 (24·3)	41 (39·8)	±2·15	6·9
Woburn	63 (52·0)	21 (27·0)	37 (37·8)	±1·76	5·6
Welshpool	38 (38·1)	25 (30·2)	33 (34·6)	±2·63	8·4
Means, 1947	56 (48·5)	22 (27·9)	36 (37·0)	±1·84	6·0
1948					
Bradninch	40 (38·9)	8 (10·9)	23 (28·0)	±4·59	14·7
Middle Cator	33 (34·5)	11 (16·6)	12 (26·4)	±5·16	16·5
Harnhill	48 (47·7)	5 (11·7)	18 (25·6)	±3·46	11·1
Welshpool	17 (24·0)	13 (20·0)	13 (21·0)	±2·58	8·3
Means, 1948	35 (36·3)	9 (14·8)	27 (25·2)	±3·56	6·46
Means, 1947 and 1948	47 (43·1)	16 (22·7)	28 (31·8)	±2·29	7·5

In the experiments sown in 1948 (group B, Table 4) the results were less uniform. Significant differences between strains were found in five of the seven experiments in which Clover F, 38 and Pentyr 4 were compared, but the relative establishment of the three strains differed strikingly in different localities, no one strain giving the lowest or highest establishment in all experiments. In the trial at Tornaveen in which strain 46 (AU) was tested, this organism gave poor establishment. Four of the experiments sown in 1947 were carried on in 1948 without resowing, and the percentage of nodules produced by each inoculum on first- and second-year plants are compared in Table 3. A considerable falling-off in establishment was apparent in the second year, but Clover **F** still gave the highest establishment.

Table 4. *Percentage of nodules containing the inoculant strains for the group B localities, with relevant standard errors (corresponding values in angular units shown in brackets)*

Group B localities, 1948	Plots inoculated with strain			S.E. (degrees)	Critical difference (5 % level) (degrees)
	Clover F	38	Pentyr 4		
Hoddesdon	25 (30·2)	37 (37·9)	23 (29·0)	±3·26	10·4
Ingatestone	43 (40·9)	12 (19·6)	51 (45·4)	±3·61	11·5
Aberystwyth	53 (46·6)	45 (42·4)	38 (37·1)	±1·93	6·2
Skipton	49 (42·6)	62 (52·1)	28 (31·5)	±2·47	7·9
Cockle Park	51 (45·3)	33 (34·5)	37 (38·0)	±3·69	11·8
Aberfoyle	24 (29·1)	50 (45·0)	48 (43·8)	±2·38	7·6
Tornaveen I	56 (50·2)	57 (46·4)	39 (38·5)	±1·82	5·8
Means omitting Tornaveen II	43 (40·7)	42 (39·7)	38 (37·6)	±3·34	10·3
	Clover F	38	46		
Tornaveen II	55 (47·7)	38 (38·3)	21 (27·4)	±2·93	9·4

The differences in establishment suggest that strains giving the best results possess some characteristics enabling them to multiply more in the root surroundings of the host crop than do other strains. The variable results in 1948 further suggest that these characteristics may be influenced by local conditions. Nevertheless, it was thought worthwhile to make a preliminary approach to this complex problem by studying the growth rates of three of the experimental strains alone and in mixture in a simple medium of sand with and without an added source of energy.

GROWTH AND COMPETITION IN SAND CULTURE

Nicol & Thornton (1941) found that when two strains of *Rh. leguminosarum* were grown together in sand surrounding the roots of growing pea plants, one strain increased in numbers and suppressed the growth of the other strain. This dominant strain also had a higher multiplication rate than the other when grown in pure culture in the root surroundings. Similar results were obtained by growing these two strains alone and together in sand supplied with sucrose but in the absence of plants. In order to learn whether the strains used throughout the establishment experiments differed in their rates of multiplication, alone and in competition with one another, the organisms were grown in sand culture with and without added sucrose and their numbers counted.

Technique. Clover F, 205 and 49 were chosen, since from the evidence of field experiments they might be expected to differ from one another in rate of multiplication. Small Erlenmeyer flasks containing 10 g. of nitrogen-deficient sand were moistened to water-holding capacity with seedling salts solution (K_2HPO_4, 0·5 %; $MgSO_4.7H_2O$, 0·02 %; NaCl, 0·01 %; $Ca_3(PO_4)_2$, 0·02 %; $FePO_4$, 0·1 %; $FeCl_3$, 0·001 %). Triplicate flasks were inoculated with a counted suspension of a 24 hr. culture of the appropriate organisms added to the solution, and the viable count estimated. Sand cultures containing 0·2 % sucrose were similarly inoculated. Cultures for a study of competition received equal numbers of each strain under test and 0·2 % sucrose; they were set up in triplicate. All cultures were incubated at 25°.

Cultures containing a single inoculum were examined after 4 days, and mixed cultures after 4 and 14 days, the time intervals being based on the data of Nicol & Thornton (1941). The numbers of bacteria were estimated by plating on calcium gluconate agar. The sand culture plus 40 ml. seedling salts solution was shaken for 10 min. before making dilutions, and five replicate plates were always poured at each dilution counted.

As colonies of the three strains used in mixed culture could not be distinguished, slide agglutination was adopted for their identification. A colony was emulsified in saline and added to a drop of the antiserum at 1/200 dilution placed on a well slide; fifty colonies from each sand culture were examined. The slides were covered with cover-slips, left at room temperature for 2 hr. and examined under the high-power objective. It was possible to distinguish agglutination from clumping due to the capsule of the organism.

Results

Growth of pure cultures. The colony counts for pure cultures, after 4 days, are shown in Table 5. The strains fall into two groups as regards growth rate: (46, Clover F, 49) > (38, 205). There was little difference in growth rate between strains within the groups but significant differences between any one strain of one group compared with any one of the other.

Table 5. *Mean colony count and bacteria/g. sand for five strains* Rhizobium trifolii *after 4 days growth in sand culture*

Strain	Inoculum	Sand	Sand and sucrose	Increase in sand	Increase due to sucrose
	Colonies and no. organisms/g. sand			(no./g. sand)	
Clover F	27·6 ± 2·91	37·0 ± 4·25	21·4 ± 2·45		
	34,000	462,000	2,675,000	428,000	2,213,000
205	22·8 ± 3·89	16·1 ± 1·85	19·5 ± 2·24		
	28,000	201,000	2,437,000	173,000	2,236,000
49	20·8 ± 4·31	32·4 ± 3·72	10·7 ± 1·23		
	26,000	465,000	1,340,000	439,000	875,000
38	69·2 ± 10·24	16·5 ± 1·89	9·3 ± 1·06		
	85,000	206,000	1,166,000	121,000	960,000
46	11·0 ± 3·63	41·3 ± 4·74	12·0 ± 1·37		
	13,000	517,000	1,500,000	504,000	983,000

In the cultures with sucrose, the groups were (Clover F, 205) > (46, 49, 38). Strain Clover F was among the fast-growing strains in each group, an interesting fact in view of its being one of the more promising to give establishment in the field. 205 showed a response to sucrose, while 38, which gave good establishment in a number of trials, grew more slowly in both media. The relative growth rates of the various strains supplied with sucrose may differ from that resulting from stimulation by the root secretion of the plant.

Table 6. *χ^2 test on colony numbers of each strain in mixed cultures to determine departure from a* 1 : 1 *ratio*

	4 days			14 days	
Strains	Cl. F	49	Cl. F		49
Culture 1	34	16	4	25	25
2	30	20	5	28	22
3	32	18	6	29	21
Total	96	54		82	68
	χ^2 (1) = 11·760			χ^2 (1) = 1·307	
	χ^2 (2) = 0·695			χ^2 (2) = 0·698	
Strains	Cl. F	205		Cl. F	205
Culture 1	18	12	4	22	28
2	29	21	5	31	19
3	32	18	6	28	22
Total	79	51		81	69
	χ^2 (1) = 6·031			χ^2 (1) = 0·960	
	χ^2 (2) = 0·386			χ^2 (2) = 3·39	
Strains	49	205		49	205
Culture 1	27	23	4	37	13
2	29	21	5	33	17
3	24	26	6	30	20
	80	70		100	50
	χ^2 (1) = 0·667			χ^2 (1) = 16·667	
	χ^2 (2) = 1·02			χ^2 (2) = 2·22	

Competition between strains. Table 6 shows the results of the identification of the fifty colonies tested from plating the 'mixed' cultures. Clover F gave a significant predominance in pairs both with 49 and 205 when sampled after 4 days' associated growth, but this predominance was no longer significant after 14 days. Such dominance may be related to the rapid growth of Clover F in pure culture by the fourth day. When 49 and 205 were grown together, the proportions of the strains did not change significantly by the fourth day, but by the fourteenth, 49 was significantly predominant. This dominance cannot be compared with growth in pure culture as no counts were made after this period. The results show that the member of each pair whose establishment in the field was higher also showed competitive dominance on one of the dates of sampling. The degree of establishment of the inoculant strains in the field did not show a clear relationship to their growth rate under standardized conditions in sand.

DISCUSSION

It is well known that a plant can bear a mixed population of effective and ineffective nodules on its root system, but the author is not aware that any estimate has been made of the proportion of effective to ineffective nodules that a plant must carry in order to receive satisfactory benefit from the former. Nutman (1946) recorded an instance in which a clover seedling received evident benefit from a single large nodule found on a root system which also carried ineffective nodules. It is probable that those who have worked in this field would agree that a plant carrying 30 % of effective nodules would benefit materially. This percentage of nodules produced by the effective inoculant strain has been taken in the present case as constituting successful establishment.

At each of the thirteen centres, at least one of the strains used for seed inoculation produced more than 30 % of the nodules, and in some cases an establishment of over 50 % was attained. The first conclusion from the trials is that it is possible to obtain successful inoculation of the crop in widely different soils which contained a varied native population of clover *Rhizobium*. It is difficult to attribute this finding solely to the dominance in competition of the inoculant strain: for it seems likely that amongst the numerous strains native to the soil, many must have possessed the ability to compete with the inoculant strains if given an equal opportunity to do so. It is possible that the placing of the inoculum on the seed-coat, from whence the bacteria can readily migrate along the root system of the seedling, gives it a critical advantage in infecting the root hairs.

Nevertheless, the second conclusion is that the nature of the strain used greatly affects the success of establishment, so that differential strain competition is an important factor. In comparing the efficacy of the different inoculant strains, the results in the 2 years show that in 1947 strain Clover F gave the best and strain 205 the lowest establishment at all the five very different localities, whereas in 1948 the strain that gave the best result differed according to locality. This was also accompanied by a greater variability in establishment between replicate plots but was not due wholly to this cause, as the effects of different strains in the same locality differed significantly in many cases. The summer of 1947 was uniformly dry, but in the wetter summer of 1948, the weather at the time of sowing varied greatly in different experiments. This factor may well have produced variable results if the inoculant strains differed in their behaviour towards the degree of soil moisture or temperature.

Inoculation practice. In the past, successful legume inoculation has been mainly confined to areas in which the soil was deficient in species of *Rhizobium* capable of infecting the crop. The present experiments indicate the possibility of extending this practice to soil known to carry ineffective strains of *Rhizobium* in Great Britain. They also indicate that the competitive dominance of strains used for inoculation must be considered in order that they may successfully replace the native flora of root-nodule bacteria in the roots of the crop. The fact that the strains used as inocula showed relative differences in different localities suggests the desirability of testing inocula consisting of several strains, in the hope that one may prove to be dominant in competition with native strains. If such mixed inocula were used, care would be necessary in selecting strains that did not compete differentially with each other.

The future of serological typing. Apart from their bearing on establishment, the experiments have shown that serological characters are a valuable aid to identification of a strain. Work with nodule bacteria has suffered because the course of infection with a given strain could not reliably be followed. When strains bearing readily identifiable antigens are chosen, the agglutination reaction forms a sound basis for their recognition and a study of antigen distribution in a native population, but separate testing for somatic and flagellar antigens was sometimes needed to distinguish a specific strain. The stability of somatic and flagellar antigens in an inoculant strain re-isolated from nodules gives confidence in the application of serological methods for recognition of specific bacterial strains in the complex environment of soil. It should be possible to recognize an inoculant strain containing a combination of two or more antigens which, though individually common, are known to occur infrequently in combination.

Note on the Statistical Examination of the Establishment Percentages

By G. V. Dyke

Department of Statistics, Rothamsted Experimental Station

To provide suitable data for analysis of variance, the percentages were transformed to degrees by the angular transformation

$$\phi^\circ = \sin^{-1} \sqrt{(p/100)},$$

where p = percentage of positives, which is appropriate for a variable following a binomial distribution. As will be observed in Table 2, the numbers of observations per plot were not constant, but the variation was relatively small and was ignored in the analysis, the transformation percentages being given equal weight.

The figures shown in Tables 3 and 4 were obtained by averaging results in angular units from each plot. The standard errors and critical differences apply to these angular values; all tests of significance were carried out on angular values. The standard errors of means for the various treatments at each site in each year were derived from analysis of results from each site and each year separately. These analyses are not presented. The standard errors of the various means of strains over groups of sites were calculated from the mean squares for the interaction 'localities by strains'.

From the analysis of variance of the group A data, it is clear that the differences in establishment with the three strains were significant. Within the limits of expected random variation, these differences were constant from year to year but varied from one locality to another. There was an obvious and considerable decrease in establishment from 1947 to 1948, and from the standard errors given in Table 3, it can be seen that the variation between plots was somewhat higher in the second year. No significant strain differences were observed at the Welshpool locality. With this exception, strain Clover F was significantly better than 205 at the 5% level throughout, but only in three cases was this strain significantly better than 49.

In the group B series Table 4 (omitting Tornaveen II) out of the seven experiments taken individually, five gave significant differences in establishment between strains; in the remaining two, at Cockle Park and Hoddesdon, these did not attain significance. The variation in establishment of each strain from locality to locality was however so great that the mean differences between strains for all localities were not significant. The group B trials thus differed from those of group A in this greater interaction between localities and strain which was accompanied by a greater variability between replicate plots.

Statistical examination of the plate counts from sand cultures

Preliminary examination of the full original data indicated that, in order to equalize the variances of groups of replicate plates with differing mean counts, the logarithmic transformation was necessary. It is likely that clumping of material produced a distribution different from the Poisson expected when the organisms are distributed independently in the diluent.

After the transformation, the data for each group of cultures were analysed in a body for each duration of growth.

The standard errors of the original mean counts were calculated by the formula

$$\text{S.E. (mean of } n \text{ cultures)} = (1/n) \times (\log_e 10) \times (\text{mean count}) \times (\text{S.E. per count in logarithmic units}).$$

The mean colony counts per strain per treatment with relevant standard errors are set out in Table 5 along with the equivalent figures as bacteria/g. Tested against the mean square between replicate cultures, the variation between strains was clearly significant, as was also the difference due to addition of sucrose to the sand. There was, however, a highly significant interaction between strains and the effect of sucrose.

After determining the proportion of colonies produced by each of the two strains in mixed cultures, a χ^2 test (1 degree of freedom) was applied to the data to determine the significance of departure from the original 1 : 1 ratio (χ^2 (1) in Table 6). All pairs showed clear departures from equality at one or other time of counting. A further calculation of χ^2 (2 degrees of freedom) was made to test the differences between replicate cultures. In no case was this difference greater than would be expected between three samples from a single population divided between two strains in the proportion indicated by the totals of the replicates.

I (M.P.R.) wish to express my gratitude to the International Federation of University Women and the Commonwealth Scientific and Industrial Research Organization, Australia, for making this research possible, and to Dr H. G. Thornton, F.R.S., for much valuable advice and criticism. I am indebted to officers of the National Agricultural Advisory Service at Leeds, Newcastle, Welshpool and Aberystwyth who carried out the nearby field trials and isolated cultures from the nodules, to Dr G. Bond for the trial at Aberfoyle, and to G. V. Dyke for the statistical analysis.

REFERENCES

Hofer, A. W. (1941). A characterization of *Bacterium radiobacter* (Beijerinck and van Delden) Löhnis. *J. Bact.* **41**, 193.

Hughes, P. Q. & Vincent, J. M. (1942). Serological studies of the root-nodule bacteria. III. Tests of neighbouring strains of the same species. *Proc. Linn. Soc., N.S.W.* **67**, 142.

Kleczkowski, A. & Thornton, H. G. (1944). A serological study of root-nodule bacteria from the pea and clover inoculation groups. *J. Bact.* **48**, 661.

Nicol, H. & Thornton, H. G. (1941). Competition between related strains of nodule bacteria and its influence on infection of the legume host. *Proc. roy. Soc.* B, **130**, 32.

Nutman, P. S. (1946). Variation within strains of clover nodule bacteria in the size of nodule produced and the 'effectivity' of the symbiosis. *J. Bact.* **51**, 411.

Thornton, H. G. (1929). The influence of the number of nodule bacteria applied to the seed upon nodule formation in legumes. *J. Agric. Sci.* **19**, 373.

Thornton, H. G. & Kleczkowska, J. (1950). Use of antisera to identify nodules produced by the inoculation of legumes in the field. *Nature, Lond.* **166**, 1118.

Vincent, J. M. (1941). Serological studies of the root-nodule bacteria. I. Strains of *Rhizobium meliloti*. *Proc. Linn. Soc., N.S.W.* **66**, 145.

Wilson, J. K. (1931). Relative numbers of two species of *Rhizobium* in soils. *J. agric. Res.* **43**, 261.

(*Received* 6 *December* 1951)

English, M. P. (1953). *J. gen. Microbiol.* 9, 15–25.

The Fermentation of Malt Extract by an Osmophilic Yeast

By MARY P. ENGLISH

Bacteriological Laboratory, Physiological Department, The British Drug Houses, Ltd., Graham Street, London, N. 1

With a Note by J. T. Gunner and Constance Rainbow on a Method of Vital Staining for *Saccharomyces rouxii*

SUMMARY: The fermentation of malt extract by the osmophilic yeast *Saccharomyces rouxii* Boutroux is described. It is found that the limiting factor determining whether an extract will ferment or not is its relative humidity, the critical level of which is about 73%. If the relative humidity is raised above this level, either by raising the moisture content or by lowering the proportion of sugars in the total solids, the extract can be fermented by *S. rouxii.*

This study of the biology of an osmophilic yeast began with the discovery of a malt extract and oil mixture which was fermenting vigorously. As this substance had a very low moisture content and contained a very high percentage of sugars, it was considered of interest to investigate the habit of any organism that could exploit conditions apparently so unpromising.

A preliminary investigation, of which a brief report has already appeared (English, 1951) showed that the chief causative organism of the fermentation was the yeast *Zygosaccharomyces japonicus* Saito now known as *Saccharomyces rouxii* Boutroux (Lodder & Kreger-van Rij, 1952), and it is this organism only with which the present work is concerned.

Though there are records in the literature of the fermentation of a large number of high-sugar substrates by osmophilic yeasts (Mrak & Phaff, 1948; von Schelhorn, 1951) no mention has so far been found of a fermentation of malt extract.

Just as there are organisms that can tolerate high sugar concentrations, so there are others which can survive in highly concentrated salt solutions and in substrates containing very low percentages of moisture. Stille (1948) found that dried foods were liable to fungal attack unless their relative humidity was less than 75%, and it is known (Joslyn & Creuss, 1929) that some yeasts can grow in pickle brine with a salt concentration of 19–20%.

The yeast named *S. rouxii* was originally found in fermenting jams and syrups, and in solid glucose, by Boutroux (1884), and in recent years has been identified by Mossel (1951) as the cause of a fermentation of sugared fruits. On the other hand, it was in strong brine, not a high sugar substrate, that the organism first given the name of *Z. japonicus* was found by Saito (1907) while he was investigating the microbiology of the products of fermenting soya in the preparation of 'koji'. *Z. japonicus* has also been recorded, with other yeasts, by Fabian & Quinet (1928) in honey, and by Fabian & Hall (1933) in maple syrup. Also Mrak, Phaff & Vaughn (1942) found that *Z. japonicus* variety *soya* was the dominant yeast in the flora of Egyptian and Californian dates.

Little is known of the effect of environment on *Z. japonicus*, but Fabian & Quinet and Fabian & Hall determined the moisture contents of fermented and unfermented samples of honey and maple syrups, and attempted to correlate these with yeast growth. Mrak *et al.* found that of all the yeasts they isolated from dates *Z. japonicus* was equalled only by two other *Zygosaccharomyces* species in its ability to grow in date syrup of 66° Balling. Mossel gives the composition and concentration of the fruits on which he found *S. rouxii*.

The work described here is a study of the effect of a number of environmental factors on the growth of *S. rouxii* in malt extract.

Description of the fermentation

It is extremely difficult to say when fermentation of a malt extract actually begins: probably the first sign is the typical sickly-sweet, slightly 'beery' smell, reminiscent of that of fermenting jam. At the same time as this smell becomes noticeable, or perhaps slightly later, individual bubbles of gas appear throughout the extract and rise extremely slowly to the surface. If the malt remains undisturbed, this stage lasts a minimum of 3–4 weeks, often considerably longer, the bubbles accumulating very slowly at the surface. At a certain point, however, the malt extract appears to become saturated with the gas, numerous minute bubbles form comparatively quickly throughout the mass, and within 10–14 days a thick layer of golden-yellow froth forms over the surface of the malt and may begin to ooze from the container. This comparatively sudden frothing can be induced quite early in the fermentation by simply stirring the malt slightly, when the same thick froth will form in 2 or 3 days, indicating that the malt becomes supersaturated with gas which can be liberated, either spontaneously when the limit of saturation is reached, or by mechanical disturbance before this point. The same phenomenon can be observed when a pure culture of the yeast is grown in a strong glucose solution. This may be stored for several days with no visible fermentation, but if it is then shaken, a mass of bubbles forms throughout the tubes some seconds, or even minutes, later.

The consistency of malt extract is such that when active fermentation is over and the yeasts are decreasing in numbers, the gas bubbles are still held by the malt, which may retain the appearance of being in a state of active fermentation for several months.

MATERIALS AND METHODS

The original fermentation was found in a malt extract and oil mixture: similar mixtures were therefore used as substrates in this work. Mixture A came from the same source as that in which the fermentation was first found, and contained 5% (w/w) of oil, while mixture B contained an extract of different composition and 10% (w/w) of oil. Mixture A was used except where B is specified. There was some variation of composition from batch to batch of both extracts, and in particular the moisture content of mixture A varied between 19·4 and 21·5 %, while that of mixture B was between 19·3 and 20·6 %.

Complete sterilization of malt extract for experimental purposes is impracticable, as the high temperatures necessary cause changes in its colour and composition. A technique of 'partial sterilization' was therefore adopted, where the extract was held at 63° for 1 hr. in a water-bath, a procedure which left the extract unchanged while killing off any fermentative organisms. Fermentation never occurred in uninoculated malt treated thus.

Small-scale experiments with malt extract were carried out in 6 × 1 in. boiling tubes, the plugs of which were covered with rubber caps to prevent excessive evaporation. For larger scale work, sterile, 450 ml. clear-glass screw-cap bottles, effectively sealed against evaporation, were used. Enough inoculum for a whole experiment was prepared by suspending yeast cells, scraped from slope cultures or centrifuged from liquid cultures, in the minimum quantity of concentrated maltose solution (so as to dilute the malt extract as little as possible) and mixing it thoroughly in about ½ lb. of yeast-free malt extract. This in turn was used to inoculate the main bulk of partially sterilized malt extract contained in a large, sterile earthenware jar, the prepared glass jars being then half-filled with the inoculated malt, sealed and incubated. All manipulations involving exposure of the extract to the air were carried out in a sterile room. Fermentation was considered to have begun when bubbles could be seen, and the typical 'fermented' smell was apparent, but it must be emphasized that, in practice, it was impossible to tell the exact time of the onset of fermentation, and times given in the results of experiments refer to stages where there was no doubt that it had started. In unstirred samples the lag period may run into weeks.

The number of yeasts in any given sample of malt extract was estimated by a modified dilution plate technique. As it had been found that temporary suspension in distilled water did not affect the viability of the yeast cells, the extract was weighed into distilled water to give a dilution of 1/10, dissolved by shaking, and 1 ml. of the solution, or of known dilutions of it, was mixed with about 7 ml. portions of an agar medium and plates poured, the colonies being counted after 4 days' incubation at 30°. The agar medium used was originally adapted from the selective medium of de Whalley & Scarr (1947) by the substitution of glucose for sucrose, but later this was found to be unnecessarily rich for *S. rouxii*, the following simplified formula proving quite satisfactory: 15 g. powdered malt extract, 0·78 g. peptone, 450 g. glucose, 15 g. powdered agar, 1 l. distilled water.

As the yeast colonies on this medium were small and difficult to count, a method of vital staining with Trypan Blue, suggested and worked out by Mr J. T. Gunner and Miss Constance Rainbow of this laboratory, was adopted as a routine. The method is described in a note at the end of this paper.

When *S. rouxii* was first isolated it was kept on a basal medium of 5 % malt agar (made from malt extract A without oil) on which it grew quickly and well.

Prolonged subculture on this medium, however, led to some loss of its osmophilic properties, and thereafter 23 % (w/w) glucose was added, and the cultures were stored at 4° and subcultured every 3 months. Inocula for most experiments were still taken from 3-day-old cultures on the basal medium, however, as one subculture on this substrate did not affect the osmophilic properties, and growth was quicker and more prolific than with the added glucose.

Cultures and experiments were incubated at 30° unless otherwise stated.

Microscopic observations on vegetative cells were made either on the living organisms or on cotton blue and lacto-phenol mounts. A satisfactory spore stain was the malachite green and safranin method (Smith, 1946) but with the omission of the alcohol wash.

Cell counts of suspensions for inoculation purposes were made on a haemocytometer.

Of several methods which were tried for the estimation of the moisture content of malt extract, a slight modification of the 'Buyze-pile' method described by Mossel & Wit (1951) proved by far the most consistent and the easiest to use. As the only drying oven available ran at 98°, drying was carried on overnight instead of for 3–5 hr.

Because of the difficulty of estimating the osmotic pressure of malt extract and heavy syrups, the related property of relative humidity was used to measure the 'physiological dryness' of the various substrates. This was determined by the usual method of exposing samples to the atmospheres of saturated solutions of various salts until equilibrium is reached, the samples being preserved from mould growth by the addition of phenyl mercuric nitrate. Equilibrium was obtained in duplicate samples in 5–6 weeks. The method was also used to adjust malt extract to known relative humidities for fermentation tests.

Isolation and identification of the fermenting organism

Isolations from the original fermented samples yielded thirty-one yeasts and several bacteria, each of which was tested for its ability to ferment malt extract. After 3 weeks incubation, twenty-four of the yeasts had fermented the substrate, while the bacteria and the remainder of the yeasts had not. Of the twenty-four fermenting yeasts isolated, twenty-two were budding yeasts, very similar to one another, and referable to the subgenus *Zygosaccharomyces*. Five of those showing the most widely divergent characteristics on malt agar were identified at the Centraalbureau voor Schimmelcultures as *Z. japonicus* Saito (now transferred to *S. rouxii* Boutroux). It could thus reasonably be concluded that *S. rouxii* was the organism chiefly concerned in the fermentation.

All the experimental work now to be described was carried out with one isolate, G1, the original culture of which was subjected to three successive single-colony re-isolations. Parallel experiments were sometimes run using other isolates, and these always gave similar results.

RESULTS

Effect of temperature on growth and fermentation

Partially sterilized malt extract was inoculated with isolate G1 at three different yeast concentrations, the precise level being measured by plate counts. The inoculated malt was filled into clear-glass jars according to the method already described, and these were incubated at 20, 25, 30, 35 and 37°. Fermentation occurred at the same time in replicates of each treatment. After 32 days, yeast counts were made on one jar from each treatment. The results are shown in Table 1.

Table 1. *The effect of temperature on the fermentation of malt extract*

Temperature	Initial yeast count (yeasts/g.)	Approximate time for the undoubted onset of fermentation (days)	Yeasts/ g. after 32 days
20°	1,515	28*	630
	17,700	28*	1,920
	462,300	28*	35,800
25°	1,515	28*	1,400
	17,700	< 28	97,000
	462,300	18	397,000
30°	1,515	< 28	43,100
	17,700	22	332,000
	462,300	10	723,000
35°	1,515	28	120,000
	17,700	< 28	398,000
	462,300	10	318,000
37°	1,515	No fermentation	1,140
	17,700	< 40†	27,000
	462,300	10	17,200

* In these cases fermentation never passed beyond the scattered bubble stage.

† Fermentation began to be apparent after the malt had been stirred for sampling (cf. p. 16).

Ingram (1949) found that in concentrated orange juice approximately 10^6 cells/ml. were necessary to cause visible fermentation. In comparison with this figure, some of those quoted for malt extract are extremely low, even taking into account that the yeast counts were made some time after the actual start of the fermentation, by which time the number of cells would be expected to be decreasing. A probable explanation lies in the physical consistency of malt extract, which is so thick that it would hold locally small bubbles of gas until enough accumulated to make a large visible bubble. In view of this, it is possible that those samples of extract in Table 1, which never reached the frothing stage, should not be considered to have fermented, in the ordinary sense of the word, even though there were demonstrably more bubbles present than in uninoculated extract.

Another possible cause for low yeast counts in the fermented extract is the plate-count method used to determine them, which was made necessary by the nature of the substrate. Though microscopic observation of the sediment from diluted centrifuged malt extract did not indicate clumping of cells to any extent, yet any clumping would of necessity reduce the apparent yeast count. The method, also, would fail to include fermenting but non-viable cells in the count.

Table 1 shows that the optimum temperature for fermentation lies between 30 and 35°; but it also shows obvious peculiarities in the behaviour of the yeast at 37°, for though fermentation is as quick as at any temperature at the highest yeast concentration, it fails to take place at all at the lowest. Malt extract darkens considerably when kept at 37° for 3 or 4 days, but no gas bubbles have been noticed during periods of storage lasting up to 3 months. The type of browning appears, therefore, to be of a different character from that of dried fruits and concentrated fruit juices stored at high temperatures, for in these products it is usually accompanied by the evolution of CO_2 (Stadtman, 1948). The darkened malt extract was not in itself toxic to *S. rouxii*, as in samples inoculated with the yeast and incubated at 30° the organism multiplied in the usual way.

For a more detailed study of the fate of the organism at 37° malt extract was inoculated with isolate G1 to give a count of approximately 12,000 yeasts/g. and was filled into glass jars and stored at this temperature. One jar was removed from storage each week for a number of weeks, and a yeast count made, the results being given in Table 2. It is clear that *S. rouxii* cannot tolerate prolonged storage at 37°, though it can continue to multiply for 4 weeks. The results shown in Table 1 can thus be explained if we consider that a small inoculum cannot multiply sufficiently in these 4 weeks to cause visible fermentation, while a larger inoculum can multiply and/or metabolize sufficiently to do so before it dies. That the sudden dying-off of the yeast after 4 weeks is not due to any toxic byproducts of fermentation is shown by the fact that the cells can survive in fermented extract at 30° for many months.

Table 2. *The effect of storage at* 37° *on the yeast count of malt extract*

Storage time (weeks)	Yeasts/g.
0	12,000
1	10,150
2	36,800
3	47,600
4*	66,300
5	7,010
6	200
7	0
8	0

* Fermentation was visible by this time in all jars.

A microscopic examination of infected malt extract which had been stored at various temperatures showed that sporing, frequently with conjugation, occurred over the whole temperature range between 4 and 37°, but that active growth was most conspicuous at 30°.

Effect of composition of the substrate on growth and fermentation

The property of a highly concentrated substrate most likely to affect critically the growth and survival of any yeast colonizing it, would be its osmotic pressure, a factor dependent both on the water content and on the relative proportion of osmotically active to osmotically inactive substances. These properties of malt extract were therefore investigated in relation to the growth of *S. rouxii*, using relative humidity as a measure of the osmotic strength of the substrate, for reasons already stated.

Heavy inoculation of samples from different batches of mixtures A and B, spread over their natural range of moisture contents, caused all samples of mixture A to ferment within 46 days, while mixture B not only failed to ferment in 2 months, but very few live yeasts were found in it at the end of this time.

The effect of adjusting the moisture contents of the two malts was then investigated. That of a sample of mixture A was reduced to 17 % by exposing it to the atmosphere of a saturated solution of potassium iodide until its weight remained constant, when an inoculum sufficiently heavy to ferment normal mixture A in a few days was added. No fermentation was apparent after 5 weeks, though the yeast was still alive at this time. In the case of mixture B, the addition of water rather than its removal was necessary. Extract adjusted to contain 22·9 % moisture began to ferment in 26 days, but that adjusted to 22·2 % moisture failed to ferment in 12 weeks. It is thus clear that, while moisture content can act as the limiting factor on the growth of *S. rouxii* in malt extract, there is no absolute value, constant from extract to extract. Some other property of malt, partly dependent on the moisture content, must be looked for.

The equilibrium relative humidities of samples of both extracts were therefore determined by the method already mentioned, and the curves obtained are shown in Fig. 1. The initial moisture contents of the samples of mixtures A and B used for this experiment were 20·9 and 20·6 % respectively, the equilibrium relative humidities as read from the graph are therefore 75·4 and 71·4 %, a difference of 4 %. The approximate limiting moisture contents for fermentation, as already determined, lie, for both extracts, at about the same value of relative humidity, 73·3 %. So that it is now possible to say (subject to the fact that only two extracts have been studied), that total osmotic pressure, expressed here as relative humidity, is the controlling factor determining whether a given sample of malt extract can be fermented by *S. rouxii*.

Now, it has already been pointed out that the total osmotic pressure of malt extract depends both on the moisture content and on the combined osmotic strength of the soluble constituents. But experiment has shown that extracts A and B have very similar moisture contents, so that this cannot account for the difference in their relative humidities, and hence in their fermentability, which must, therefore, lie in the total osmotic pressures of the soluble constituents. As it proved difficult to obtain an accurate analysis of the solids of the two malt extracts, and so to compare them directly, it was decided instead to measure and compare their total osmotic strengths through the property of depression of the freezing-point of water, and Dr R. E. Stuckey, of the Analytical Department, kindly undertook this estimation. The results, expressed in terms of the equivalent percentage (w/w) of glucose, were as follows:

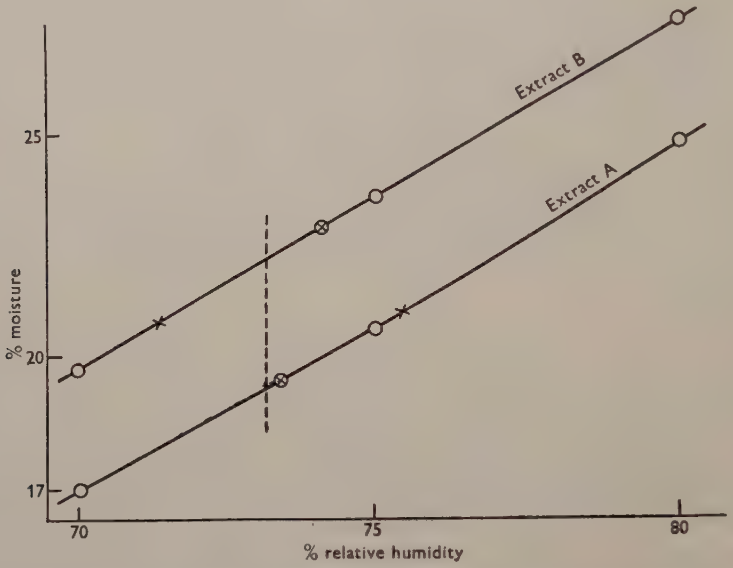


Fig. 1. Moisture content and relative humidity of malt extract mixtures *A* and *B*. ⊗, lowest moisture content at which mixture is known to ferment; ×, relative humidity of the untouched mixture. The broken line indicates approximately the relative humidity below which neither mixture can be fermented by *S. rouxii*.

Mixture	Depression of freezing point (°)	Equivalent % glucose
A	1·165	39·9
B	1·319	47·6

thus confirming the view that it is the difference in osmotic strength of the ingredients that is the cause of the difference in fermentability of the two mixtures.

The general conclusion can now be drawn, that *S. rouxii* cannot ferment a malt extract with a relative humidity of 73 % or less, and that the relative humidity can be adjusted either by altering the moisture content or the proportion of sugars to non-sugars in the solids content.

DISCUSSION

Fabian & Hall (1933) attempted to find the exact level of moisture content at which artificially inoculated honey would begin to ferment, by standing the infected honey in a saturated atmosphere and determining the moisture content when the first visible signs of fermentation appeared. But we know (Kroemer & Krumbholz, 1931; von Schelhorn, 1950) that visible fermentation in highly concentrated media takes a long time to set in, probably (Ingram, 1949) due to slow multiplication of the yeast; so that with a continually increasing moisture content, as in Fabian & Hall's experiment, fermentation could well have taken place at moisture levels below those actually recorded if the honey had remained long enough at these levels. In fact, after describing this experiment, in which the most concentrated honey used contained 32·8 % moisture at first, but did not ferment visibly until it contained 34·1 %, Fabian & Hall mention that a desiccated honey containing 'about 10 %' of moisture was slowly fermented when inoculated with their 'best fermenting yeast'. It seems, therefore, that in this experiment the authors are measuring the time taken for visible fermentation to set in, rather than the lowest moisture level at which it is possible.

If *S. rouxii* is compared with other xerophilic and osmophilic organisms for which figures are available, it is seen to be somewhat less resistant than many of them. Stille (1948) found, for instance, that *Aspergillus glaucus* could produce spores at a relative humidity of 70 %, a level which kills *S. rouxii* in 2 months, while von Schelhorn (1950) has shown the extraordinary resistance of selected strains of *Z. barkeri* which can grow slowly in fructose syrups of 62 % relative humidity. It is possible that with careful selection and 'training' a more resistant strain of *S. rouxii* could be obtained. Though Fabian & Quinet (1928), Fabian & Hall (1933) and Lochhead (1933) gave limits of moisture content below which naturally infected honey and maple syrup would not ferment, they did not give the relative humidities of the substrates, making it difficult to compare their results with others. The results described in this paper agree with those of other investigators (see Mossel & Westerdijk, 1949) in demonstrating that in systems of high sugar (or salt) content the controlling factor for the growth of yeasts is not the absolute moisture content, but the availability of the moisture present as measured by the osmotic pressure or relative humidity of the substrate. They show more clearly than most, however, the exact relationship between the sugar and moisture content of the system in their effect on the final relative humidity. The recent work of Schachinger (1950) bears directly on this point. He shows that for bakers' yeast growth ceases completely in 47 % glucose or fructose (monosaccharide) solutions, but in sucrose (disaccharide) syrup, it can continue up to a concentration of 60 %. All these solutions have a relative humidity of about 90 %. For osmophilic yeasts the same effect was noted, but the limiting sugar concentrations were considerably higher.

In the case of malt extract, for equal moisture contents, the samples most likely to ferment would be those that had undergone the least hydrolysis during the manufacture of the malt, and hence would have comparatively high dextrin/maltose and maltose/glucose ratios and correspondingly high relative humidities. Samples which had undergone more complete hydrolysis and contained comparatively high proportions of sugars, especially glucose, would be those likely to inhibit yeast growth. An examination of different malt extracts with this observation in mind would be of interest.

My thanks are due to Dr Kreger van Rij of the Centraalbureau voor Schimmelcultures, who identified the original yeast isolates for me, and Miss Sloof, who advised on their nomenclature: to Dr C. J. Hickman of the University of Birmingham, for his criticism of the manuscript, and to Dr C. Rainbow of the same University, who also read the manuscript: to Dr S. W. F. Underhill of this Department for his continued encouragement; and to the Directors of The British Drug Houses Ltd. for permission to publish these results. Most particularly, I should like to thank Mr J. T. Gunner and Miss Constance Rainbow for their technical assistance.

REFERENCES

Boutroux, M. L. (1884). Sur la conservation des ferments alcooliques dans la nature. *Ann. Sci. nat.* (*Bot.*), **6**, 144.

English, M. P. (1951). Fermentation of malt extract. *Nature, Lond.* **168**, 391.

Fabian, F. W. & Hall, H. H. (1933). Yeasts found in fermented maple syrup. *Zbl. Bakt.* (Abt. 2), **89**, 31.

Fabian, F. W. & Quinet, R. I. (1928). A study on the cause of honey fermentation. *Tech. Bull. Mich. agric. Exp. Sta.* **48**, no. 92.

Ingram, M. (1949). Fermentation in concentrated orange juice. *Food Manuf.* **24**, 77.

Joslyn, M. A. & Creuss, W. V. (1929). A comparative investigation of certain film-forming fungi. *Hilgardia*, **4**, 201.

Kroemer, K. & Krumbholz, G. (1931). Untersuchungen über osmophile Sprosspilze. I. *Arch. Mikrobiol.* **2**, 352.

Lochhead, A. G. (1933). Factors concerned with the fermentation of honey. *Zbl. Bakt.* (Abt. 2), **88**, 296.

Lodder, J. & Kreger van Rij, N. J. W. (1952). *The Yeasts, a Taxonomic Study.* Amsterdam: Noord-Hollandsche Uitgeversmij.

Mossel, D. A. A. (1951). Investigation of a case of fermentation in fruit products, rich in sugars. *Leeuwenhoek ned. Tijdschr.* **17**, 146.

Mossel, D. A. A. & Westerdijk, J. (1949). The physiology of microbial spoilage in foods. *Leeuwenhoek ned. Tijdschr.* **15**, 190.

Mossel, D. A. A. & Wit, S. L. (1951). Determination of the water content of foods. III. *Analyt. Chim. acta*, **5**, 63.

Mrak, E. M. & Phaff, H. J. (1948). Yeasts. *Annu. Rev. Microbiol.* **2**, 1.

Mrak, E. M., Phaff, H. J. & Vaughn, R. H. (1942). Yeasts occurring on dates. *J. Bact.* **43**, 689.

Saito, K. (1907). Mikrobiologische Studien über die Soyabereitung. *Zbl. Bakt.* (Abt. 2), **17**, 20.

Schachinger, L. (1950). Osmotischer Druck und Mikroorganismenwachstum auf Lebensmitteln. *Z. LebensmittUntersuch.* **91**, 423.

Schelhorn, M. von (1950). Untersuchungen über den Verderb wasserarmer Lebensmittel durch osmophile Mikroorganismen. I. *Z. LebensmittUntersuch.* **91**, 117.

Schelhorn, M. von (1951). Control of micro-organisms causing spoilage in fruit and vegetable products. *Advanc. Food Res.* **3**, 429.

Smith, G. (1946). *An Introduction to Industrial Mycology.* London: Arnold, p. 56.

STADTMAN, E. R. (1948). Nonenzymatic browning in fruit products. *Advanc. Food Res.* **1**, 325.

STILLE, B. (1948). Grenzwerte der relativen Feuchtigkeit und des Wassergehaltes getrockener Lebensmittel für den mikrobiellen Befall, *Z. LebensmittUntersuch.* **88**, 9.

WHALLEY, H. C. S. DE & SCARR, M. P. (1947). Micro-organisms in raw and refined sugar and intermediate products. *Chem. & Industr.* **66**, 531.

Note on a Method of Vital Staining for *Saccharomyces rouxii*

By J. T. GUNNER and CONSTANCE RAINBOW

A number of dyes were investigated as possible vital stains for *S. rouxii*, and of these Trypan Blue proved the most satisfactory. After some trial, the following method was found suitable for the routine counting of yeast colonies in dilution plates.

A 0·1 % solution of Trypan Blue in sterile distilled water was boiled for 5 min. and kept aseptically as stock. When required, enough of this solution was added to the tubed, melted agar medium to give a final concentration of 1/10,000 Trypan Blue, and plates were poured.

The stain at this concentration was entirely nontoxic to *S. rouxii*, which absorbed it readily to give very dark blue colonies easily visible against the light blue of the agar.

(*Received* 31 *October* 1952)

Bergersen, F. J. (1953). *J. gen. Microbiol.* 9, 26–29.

A Probable Growth Cycle in *Bacillus megaterium*

By F. J. BERGERSEN

Department of Bacteriology, University of Otago Medical School,
Dunedin, New Zealand

SUMMARY: The sequence of events which occurs as *Bacillus megaterium* grows and divides is shown to involve cells with fusion nuclei and cells which undergo an apparently sexual process. An account is also given of the part played in cell division by granules associated with the cell membrane. These resemble mitochondria in that they are centres of intense oxidative activity, and they are shown to be related to growing points which have been reported in other organisms.

Life cycles have been described in many micro-organisms, and much work has been done in interpreting observed processes; this has been well summarized and reviewed (Bisset, 1950, 1951*a*). There have been recent papers by Mudd, Winterscheid, DeLamater & Henderson (1951) and Mudd, Brodie, Winterscheid, Hartman, Beutner & McLean (1951), which describe cytochemical and electron microscope investigations into the existence of mitochondria in bacteria. These have revealed granules of high oxidative activity in certain aerobic organisms. Bisset (1951*b*), using ordinary stains, has shown that some bacteria have regions of more active growth, which he termed growing points.

I have found that growing points can be demonstrated in HCl-Giemsa preparations of many species of bacteria (Bergersen, 1952). Bisset (1953) has shown that the appearances described by Mudd *et al.* (1951*a*, *b*) are merely growing points and often entire distorted cells. It was with the object of further investigation of the two views that the present work was undertaken.

MATERIALS AND METHODS

The organism used was a strain of *Bacillus megaterium*, isolated and identified in this laboratory. It was maintained on nutrient agar. Cultures for investigation were made in a meat infusion broth, incubated at 37° and examined after various time intervals. HCl + Giemsa preparations were made on coverslips, smears being fixed in osmic acid vapours and hydrolysed at 60° in N-HCl. Living cultures growing on a 0·5 % agar medium contained under coverslips in hollow slides were examined by phase microscopy. Such preparations were observed at intervals, the microscope, with the slide in position, being placed in a 37° incubator.

Cytochemical reagents used were Janus Green B, and Nadi reagent which was prepared before use by mixing 1 vol. of 1 % α-naphthol in 95 % ethanol with 1 vol. of 1 % aqueous dimethyl-*p*-phenylenediamine, and filtering the mixture.

OBSERVATIONS

The HCl+Giemsa preparations revealed an orderly series of events. The inocula were taken from actively growing broth cultures which consisted of chains of bacilli with paired nuclear structures and growing points at the poles of each cell (Pl. 1, fig. 1).

After 3 hr. the culture contained elongated cells with bars of chromatinic material (fusion nuclei) (Pl. 1, fig. 2). The lateral cell membranes were studded with three or four growing points, and where the cells were dividing these were often large. Also present were a number of cells with two isolated chromatinic bodies, a number of apparently anucleate cells, and in some microscope fields cells were found which were fusing and apparently transferring nuclear material. The last three types of cells did not contain growing points.

Eight hours after inoculation the bacilli were elongated and in chains of three or four (Pl. 1, fig. 3). The nuclear bodies were still rods and growing points were present in lateral cell membranes and at polar positions.

After 10 hr. these long bacilli began to break up into cells of the same size as those of the inoculum, and the nuclei appeared as zig-zags of short rods in each cell (Pl. 1, fig. 4). Growing points were mainly at transverse septa but some were still present in lateral cell membranes.

Twelve to fifteen hours after inoculation the culture consisted of typical anthracoid filaments, whose cells were of the same type as those of the inoculum (Pl. 1, fig. 5).

These observations suggested that the growing points associated with the cell membrane were concerned in cell division. If this be so they appeared before division actually began.

Phase-contrast studies showed granules in the cell periphery in both lag and logarithmic phases; during active multiplication they were obscured. These granules seemed to occupy positions at the cell membrane corresponding to the growing points (Pl. 2, fig. 6). Dividing organisms were seen to have the granules at the site of division, and in fact division was not observed in their absence provided that they were unobscured by the processes of rapid growth. By limiting the oxygen supply in slide cultures by sealing with wax, growth was halted in the late lag phase; the organisms then showed the granules typical of that stage of growth. When oxygen was again allowed to diffuse into the culture division occurred in the regions of the granules.

A late lag phase slide culture showing these bodies was photographed with the phase microscope, and then Nadi reagent was flooded under the coverslip. After 2·5 hr. incubation with this solution, the same field was photographed with ordinary bright field illumination (Pl. 2, fig. 7). The granules appeared in identical positions and stained a deep blue, indicating that they contained relatively large amounts of cytochrome oxidase. Similar work, using Janus Green B, confirmed the oxidation-reduction activity of the granules which appear in phase-contrast preparations. It seems likely that the growing points seen in stained specimens correspond in some way with these granules.

DISCUSSION

The growing points of *B. megaterium* have some of the properties of mitochondria in that they are centres of oxidation-reduction processes involving cytochrome oxidase. They appear at the site of cell division before this takes place. It is possible that the bodies appearing in HCl+Giemsa preparations, and referred to as growing points, are not the granules themselves, but either densely staining material around them or their remains after partial destruction by hydrolysis. It is not surprising that the vigorous processes of bacterial cell division involve organelles which supply the requisite energy for the chemical syntheses involved. Watson (1952) has shown with rat testis cells that mitochondria are associated with the cell membrane of spermatids. These cells are also in a state of rapid development and division.

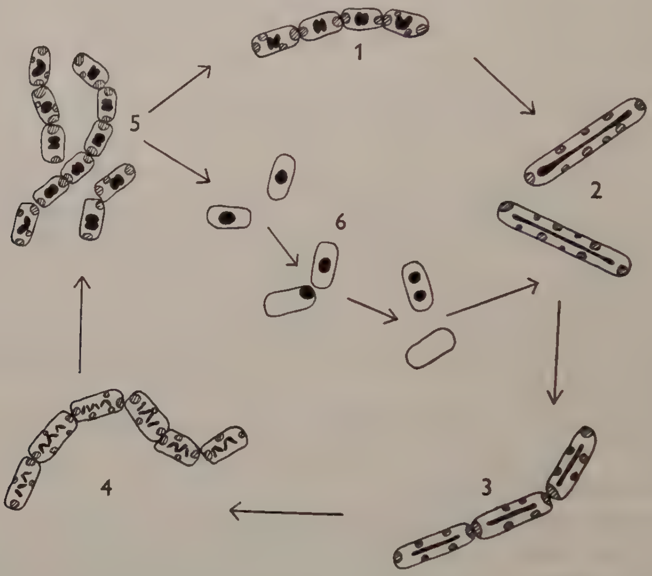
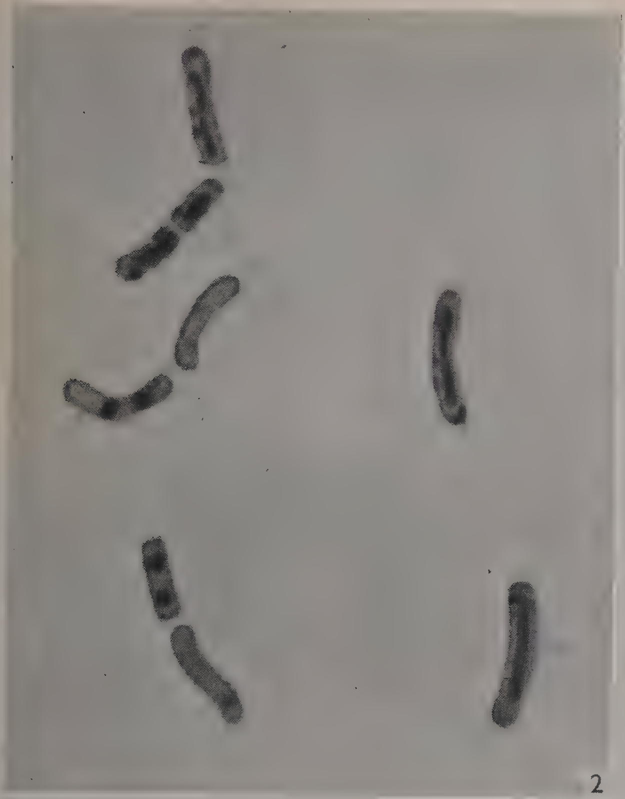


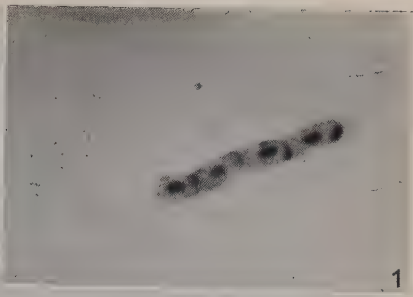
Fig. 1. (1) the appearance of the actively growing inoculum; (2) elongated cells with fusion nuclei, and peripheral growing points; (3) a later stage than (2); (4) cells in chains, nuclei in zig-zag rods; (5) a mature culture; (6) suggested sexual phase.

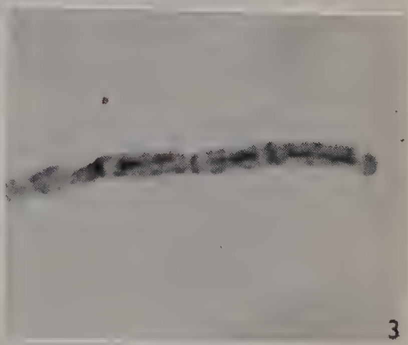
Nuclear bodies are blocked in and growing points are cross-hatched.

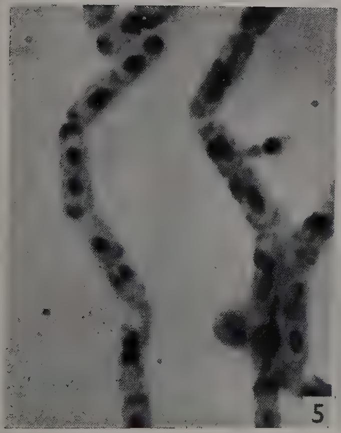
The behaviour of the nuclei during the life cycle of *B. megaterium* does not differ from that observed with many other species of *Bacillus*. There is a fusion of chromatinic material before vegetative division and a probable sexual stage involving morphologically distinct gamete-like cells (Fig. 1).

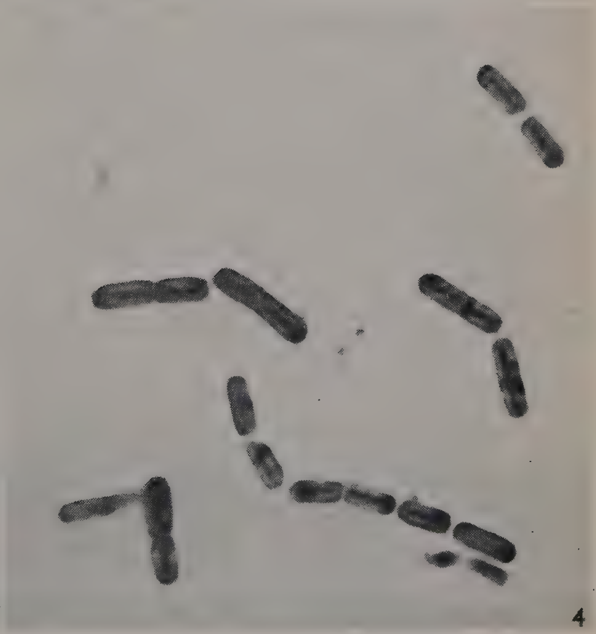
I wish to thank Dr M. J. Marples for advice and for correcting the manuscript.


2

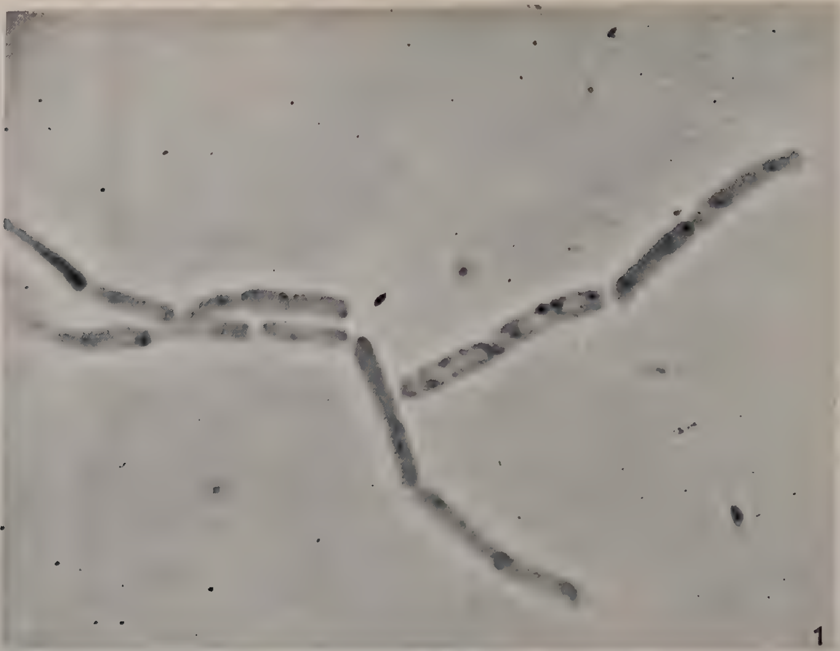

1


3

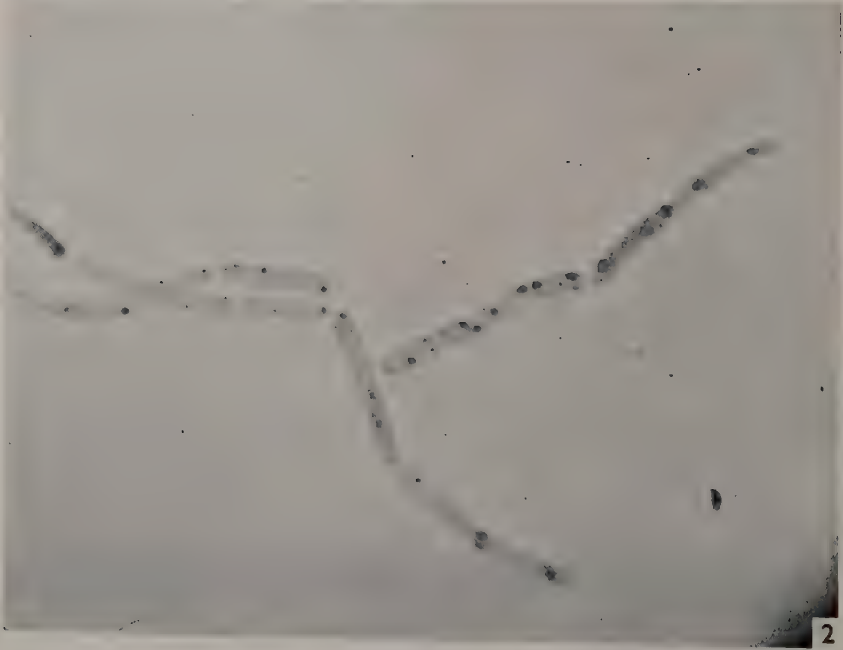

5


4

F. J. BERGERSEN—GROWTH CYCLE OF *BACILLUS MEGATERIUM*. PLATE 1



1



2

F. J. BERGERSEN—GROWTH CYCLE OF *BACILLUS MEGATERIUM*. PLATE 2

REFERENCES

BERGERSEN, F. J. (1952). Cytological changes induced in *Esch. coli* by chloramphenicol. *Proc. Univ. Otago med. Sch.* **30**, 3.

BISSET, K. A. (1950). *The Cytology and Life History of Bacteria.* Edinburgh: E. and S. Livingstone Ltd.

BISSET, K. A. (1951*a*). The morphology and cytology of bacteria. *Annu. Rev. Microbiol.* **5**, 1.

BISSET, K. A. (1951*b*). The development of the surface structures in dividing bacteria. *J. gen. Microbiol.* **5**, 155.

BISSET, K. A. (1953). Do bacteria possess mitotic spindles, fusion tubes and mitochondria? *J. gen. Microbiol.* **8**, 50.

MUDD, S., WINTERSCHEID, L. C., DELAMATER, E. D. & HENDERSON, H. J. (1951*a*). Evidence suggesting that the granules of mycobacteria are mitochondria. *J. Bact.* **62**, 459.

MUDD, S., BRODIE, A. F., WINTERSCHEID, L. C., HARTMAN, P. E., BEUTNER, E. H. & MCLEAN, R. A. (1951*b*). Further evidence for the existence of mitochondria in bacteria. *J. Bact.* **62**, 729.

WATSON, M. L. (1952). Spermatogenesis in the albino rat revealed by electron microscopy. *Biochim. biophys. Acta*, **8**, 369.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Cells of the inoculum showing paired chromatinic bodies and polar growing points. HCl+Giemsa. ×1500.

Fig. 2. Bacilli from a 3 hr. culture, showing elongated cells with fusion nuclei and lateral and terminal growing points. Note also anucleate cells and others of the sexual cycle. (See Fig. 1.) HCl+Giemsa. ×1500.

Fig. 3. Organisms from an 8 hr. culture, still containing fusion nuclei and with cell membranes studded with granules. HCl+Giemsa. ×1500.

Fig. 4. A 10 hr. culture. Fragmentation of the elongated cells has commenced and the nuclear material is in a zig-zag of rods in each cell. HCl+Giemsa. ×1500.

Fig. 5. A mature 15 hr. culture. HCl+Giemsa. ×1500.

PLATE 2

Fig. 6. Phase-contrast photomicrograph of late lag phase cells of *Bacillus megaterium*, showing the peripheral granules. ×1500.

Fig. 7. The same cells as Fig. 6 after treatment with Nadi reagent for 2½ hr. ×1500 (ordinary bright field illumination).

(*Received* 10 *November* 1952)

Newcombe, H. B. (1953). *J. gen. Microbiol.* 9, 30–36.

Radiation-induced Instabilities in Streptomyces

By H. B. NEWCOMBE

Atomic Energy of Canada Limited, Chalk River, Ontario, Canada

SUMMARY: A variety of heritable changes affecting colony morphology and colour are induced in a *Streptomyces* sp. by exposure of the spores to ultraviolet and gamma rays. Most of the changes are associated with instabilities which give rise to further variation during colony growth and spore formation. The instabilities persist indefinitely in most lines of descent and give rise to characteristic proportions of new variant types each having its own pattern of instability. The ultraviolet dose/effect curve shows the familiar peak and decline found for mutations in many other organisms. The present changes, however, differ from the gene mutations previously studied in that they can be induced with much greater frequencies (30–60 % of the colonies being affected), and that gamma rays are as effective or more so than ultraviolet irradiation. This suggests that the initial changes, and perhaps subsequent ones as well, might be more of the nature of the chromosomal rearrangements, rather than of the gene mutations, of higher organisms. Similar instabilities arise spontaneously but with a much lower frequency.

Studies of sectoring for induced mutations in the colonies from irradiated bacteria have been complicated by the lack of precise information as to the number of nuclei in the irradiated cells (Newcombe, 1951, 1953; Witkin, 1951*a*, *b*). To avoid this difficulty an actinomycete, the spores of which were known to be uninucleate (J. McGregor, unpublished; see also Klieneberger-Nobel, 1947), was chosen for the further study of induced sectoring.

It is known that ultraviolet- and gamma-irradiated actinomycete spores yield high proportions of mutant colonies, and that some of the changes occur as sectors (Kelner, 1948, 1949), but the sectoring phenomenon had not been studied in any detail. The present observations show that the spontaneous and induced mutations occurring in the actinomycetes have a very different behaviour from those studied previously in the bacteria.

Materials and methods

A strain of *Streptomyces* sp. was supplied by Dr J. W. Rouatt (his strain T12), the colonies of which have a deep orange-coloured base covered with a fluffy layer of paler orange aerial mycelium. All platings were on a medium composed of: asparagine, 0·05; peptone, 0·05; $K_2HPO_4.3H_2O$, 0·05; glucose, 1·0; agar, 1·5 (as %, w/v). Spore suspensions were prepared by flooding the confluent growth on agar with normal saline (0·85 %), scraping and agitating to loosen the spores, decanting, blending for 30 sec., and filtering through No. 2 Whatman filter-paper to remove any pieces of aerial mycelium. Suspensions could be stored in the cold for a month or more with very little loss of viability.

The treatments were carried out with gamma rays from Cobalt 60 (administered at the rate of 5000 r./hr.) and with ultraviolet irradiation by a G.E.

Germicidal lamp. In the case of the ultraviolet irradiation 10 ml. of spore suspension were exposed in a 10 cm. diameter Petri dish, with continuous agitation. The dose of gamma radiation was determined chemically (using a ferrous salt system), and ultraviolet dose by means of a Westinghouse dose-meter.

Types of change

Changes involving loss of aerial mycelium were classed as: *crinkled* (an uneven decrease giving the colony a dimpled appearance at the centre with narrow radial valleys reaching to the edge); *stubble* (incomplete loss); and *waxy* (complete loss). Losses of the orange pigment of the base resulted in either a *yellow* or a *white* colour. Areas with altered aerial mycelium often had altered colour as well. Each of the above categories contained a wide range of types differing from one another in such minor properties as tint and texture of the aerial mycelium, and its distribution over the surface of the colony. Of the *stubbles*, for example, some had a darker orange and some a paler orange stubbling; the density of the stubbling was sometimes greatest at the centre, sometimes greatest at the periphery, and sometimes uniform.

The above categories of apparent losses include all but the rarer changes, such as those to a smaller colony size (*minute*) or to a *pink* or *red* base, together with some alterations of the texture and shade of the aerial mycelium which were too slight to be scored with accuracy. Peripheral sectors which obviously arose during the growth of otherwise normal colonies were not scored.

Sectoring of a colony for two or more types of change, or for different degrees of the same change, was common both with ultraviolet and with gamma irradiation (see Table 1), but sectors of normal appearance were relatively infrequent (17·7 % of the 514 gamma-induced mutants had sectors which looked normal) and almost all yielded spores which developed into variant colonies. (For examples of the colony types see Pl. 1, figs. 1–5.)

Table 1. *Multiplicity of sectoring in colonies from irradiated spores*

	No. of colonies			
	Gamma* treated Colonies		Ultraviolet† treated Colonies	
No. of types of growth in a single colony	No.	%	No.	%
One (normal)	1158	69·3	927	83·1
One (mutant)	142	8·5	82	7·4
Two	225	13·4	58	5·2
Three	114	6·8	44	3·9
Four	33	2·0	4	0·4
Total colonies	1672	—	1115	—
Mutant colonies	514	—	188	—

* 50,000 r; survival approx. 0·1 %.

† Combined data from 1000 and 2000 ergs/sq.mm.; survivals approx. 0·017 and 0·006 %.

Instability

Since the irradiated spores were known to be uninucleate and free from appreciable clumping (as indicated by direct observation and by the extreme rarity of genuine normal sectors) the multiplicity of sectoring must indicate some instability during colony growth. A further and apparently greater instability during spore formation was indicated by the range of variant colony types which grew from spores of the morphologically normal sectors, and also from those of the *crinkled* and *stubble* areas.

Sectoring. Early changes must be assumed to have occurred in some of the colonies where the sectors were regular and extended to the centre. Prolonged instabilities seem, however, to have been common since shallow sectors of *stubble* and *waxy* were frequent, and were often surrounded by the *crinkled* type from which they appeared to have arisen. *Yellow* (and *white*) appeared sometimes to have been derived in turn from *stubble* and *waxy*, and sometimes directly from *crinkled.*

Further support for the concept of *crinkled* as a category of primary unstable types was provided by: (1) its high frequency of occurrence (all but 23 % of the gamma-induced mutant colonies had crinkled areas); (2) its appearance of inhomogeneity; (3) the tendency for the other categories of change to occur preferentially in colonies with crinkled areas (see Table 2).

Table 2. *Correlated occurrence of* crinkled *in the same colony with other types of change*

Dose of gamma radiation 50,000 r.

Types of variants (other than crinkled)	Colonies			
	Without crinkled areas		With crinkled areas	
	No.	%*	No.	%†
No other type	1158	—	136	—
Aerial mycelium only‡	83	6·5	118	29·7
Colour only§	7	0·6	17	4·3
Aerial mycelium and colour‖	27	2·2	126	31·7

* Of colonies without crinkled.
† Of colonies with crinkled.
‡ Stubble or waxy.
§ White or yellow.
‖ In the same colony, not necessarily the same sector.

The concept of *stubble* and *waxy* as secondary unstable types from which an appreciable proportion of the changes to *yellow* and *white* were derived is supported by the frequencies with which the *yellow* and *white* areas were themselves *stubble* or *waxy*, and with which they occurred together in the same colony with *stubble* or *waxy* areas of normal colour (see Table 3). It seems probable that there are many primary unstable states giving rise to variant sectors and spores, and that these variants are themselves unstable, and so on indefinitely. Certainly this was true of the instabilities which became apparent during spore formation.

Table 3. *Correlated occurrence of colour variations with* stubble *and* waxy

Dose of gamma radiation 50,000 r.

	Absence or presence of stubble or waxy areas elsewhere in the colony		
	Absent (no.)	Present (no.)	Percentage (%)
Colonies with colour variations			
Fluffy yellow area*	24	11	32·3
Stubble yellow area*	50	44	46·6
Waxy yellow area*	18	39	68·4
All colonies†	1318	354	21·2

* Or white.
† Irrespective of colour variations.

Unstable lines. A few of the variant types were stable on subculture, but the majority yielded spores which developed into a further range of variant colonies, reversions to a stable wild-type appearance being rare. Spores from the morphologically normal sectors of induced mutant colonies, and from *crinkled* areas, gave rise to mixtures of various 'mutant' types of growth (some of them occurring as sectors) and of morphologically normal (or nearly normal) colonies carrying the instability. These latter made up 15–75 % of the total colonies, and spores from them grew in turn into a similar mixture of colony types, and so on. Thus certain of the unstable lines could be perpetuated indefinitely by serial substreaking from the spores of successive morphologically normal colonies. Such lines differed among themselves in the appearance of the various colony types and in the proportions of the different categories. Thus, of four unstable lines studied in detail, line 1 gave approximately 60 % morphologically normal colonies at each transfer and line 2, 20 %. The *crinkled* category was common in the streaks of lines 1 and 4, but rare in those of 2 and 3. *Waxy* was common in lines 2 and 3, and *yellow* (or *white*) *waxy* areas occurred with frequencies of about 10 % in line 2 and 1 % in line 3. All *waxy* types were rare in lines 1 and 4, but *fluffy white* areas occurred occasionally and were not seen in the other lines. The crinkled colonies from line 3 were *minute* except for 1 or 2 % which were of normal size. (Pl. 1, fig. 6 shows the colonies which grew from spores of unstable line 2.)

Where substreaking was always from spores of successive morphologically normal (or nearly normal) colonies of precisely the same appearance, the proportions of the various colony types tended to remain the same; line 2, for example, showed no change over fifteen transfers. However, unstable lines which yielded new colony types, or altered proportions, were obtained by subculturing from colonies of a different appearance, and an apparently limitless collection of unstable lines could be derived by successive selections of different variants.

The absence of peripheral waxy sectors in the morphologically normal colonies of unstable line 2 is at first sight difficult to reconcile with the higher proportion of waxy spores from these colonies. However, at least two hypotheses are possible: (1) nuclear instability might be limited to the times of spore germination and of spore formation (or formation of the aerial mycelium); or (2) genetically waxy nuclei might arise at a constant rate per nuclear division to produce a heterokaryon with a nearly normal phenotype. In the latter case mutations to *waxy* which occurred in the early nuclear divisions might result in *waxy* sectors, with later mutations contributing only to the heterokaryosis. It should be noted that with either of the above interpretations, high degrees of nuclear instability must be assumed.

Stable lines of some of the various 'mutant' types were found occasionally. Also, stable lines which were indistinguishable from the wild type, were obtained from certain rare colonies of line 2. As the usual 'morphologically normal' colonies of this line had aerial mycelium with a slightly stronger orange tint than the wild type, the few stable colonies could be distinguished. It is of interest that the return to normal stability was coupled in these cases with a return from a nearly normal tint of aerial mycelium to what was apparently the precise wild type. All changes seemed to occur in a stepwise manner, and a number of attempts to select for gradual shifts in the proportions of morphologically normal colonies failed. However, there was no predetermined sequence of changes; nor were the changes a matter of irretrievable loss of characters, since in the case of one of the *stubble yellow* lines a proportion of the spores developed into colonies of the normal colour (*stubble orange* and *waxy orange*).

Thus, the overall pattern of the instabilities appeared to be of almost limitless complexity, both the likelihood of further change and the directions which it could take differing in different lines and changing with each change in colony morphology; there appeared to be a limitless range of phenotypes. Occasionally variant colonies arose from untreated wild type spores, and these were indistinguishable from the induced variants and gave rise to similar unstable lines.

Nature of the induced changes

The initial changes in Streptomyces had two properties in common with those of many other organisms: they were induced by ultraviolet and ionizing radiations, and the ultraviolet dose/effect curve reached a peak followed by a decline (see Table 4). By analogy, these changes might be expected to be due to nuclear events, but they differed from the gene mutations of other organisms in that they were induced in much higher proportions, and that gamma irradiation was equally or more effective than was ultraviolet irradiation. This is the behaviour which might be expected if gross chromosomal rearrangements were predominant. Such an interpretation, in view of the apparent similarity between the initial induced changes and those which followed spontaneously, would follow from the fact now well established in at least one higher organism (see McClintock, 1951), that certain chromosomal rearrangements can cause a predisposition to further rearrangement (and perhaps to gene mutation). And, if one wishes to speculate further, McClintock's (1951, p. 43) suggestion, that alterations in heterochromatin could be responsible for the development of unstable lines in maize, might perhaps be applicable also to the present organism. No attempt will be made to discuss alternative interpretations, except to indicate that any which involve a segregation of pre-existing genetic particles as the sole cause would not seem to account satisfactorily either for the onset of a supposed ability to segregate or for the apparently limitless variety of phenotypes. Unstable lines have, of course, been described in many organisms (for a recent and closely parallel case in *Bacterium coli*, described initially by Braun and Lewis, see Bryson, Prytz, Cuneo, Yongen & Raymond, 1950), and the Streptomyces case may not be unique except in the ease with which the instabilities can be induced, and in the number of variant types which can be recognized.

Table 4. *Frequency of mutant colonies from irradiated and non-irradiated spore suspensions*

Treatment	No. colonies examined	Mutant colonies No.	Mutant colonies %
None (control)	5507	29	0·5
Gamma 50,000 r.	1672	514	30·7
Ultraviolet (ergs/sq.mm.)			
500	357	118	33·1
1000	518	89	17·2
2000	587	89	15·2

Note. The above experiment is typical of many which have been carried out. In some the gamma irradiation produced approximately the same proportions of mutant colonies as the ultraviolet, and in others it resulted in as much as twice the proportion (up to 60 % mutant colonies).

I should like to acknowledge the technical assistance of Mr J. McGregor.

REFERENCES

Bryson, V., Prytz, B., Cuneo, H., Yongen, E. & Raymond, C. (1950). Morphological and biochemical analysis of resistant bacteria. *Annu. Rep. Biol. Lab. Cold Spring Harbor*, no. 20.

Kelner, A. (1948). Mutation in *Streptomyces flaveolus* induced by X-rays and ultraviolet light. *J. Bact.* **56**, 457.

Kelner, A. (1949). Studies on the genetics of antibiotic formation: the induction of antibiotic-forming mutants in *Actinomycetes*. *J. Bact.* **57**, 73.

Klieneberger-Nobel, E. (1947). The life cycle of sporing actinomyces as revealed by a study of their structure and septation. *J. gen. Microbiol.* **1**, 22.

McClintock, B. (1951). Chromosome organization and genic expression. *Cold Spr. Harb. Sym. quant. Biol.* **16**, 13.

Newcombe, H. B. (1951). Sectoring in colonies from irradiated cells of *Escherichia coli*. *Genetics*, **36**, 570.

Newcombe, H. B. (1953). The delayed appearance of radiation induced genetic change in bacteria. *Genetics*, **38**, 134.

Witkin, E. (1951*a*). Nuclear segregation and delayed expression of induced mutations in *Escherichia coli*. *Genetics*, **36**, 583.

Witkin, E. (1951*b*). Nuclear segregation and delayed appearance of induced mutants in *Escherichia coli*. *Cold Spr. Harb. Sym. quant. Biol.* **16**, 357.

EXPLANATION OF PLATE

Figs. 1–5 are of colonies from irradiated spores.

Fig. 1. Three normal colonies (left); two wholly *crinkled* (right).

Fig. 2. A wholly *stubble* colony (except for central tuft of fluffy aerial mycelium) showing sectoring for different degrees of loss of aerial mycelium and different growth habits.

Fig. 3. A wholly *waxy* colony (left) showing a yellow sector at 1 o'clock, and a sector with slight colour change at 5 o'clock. A *crinkled* colony (right) with two large *stubble* sectors at 4 and 7 o'clock, and two small *waxy* sectors at 11 and 1 o'clock.

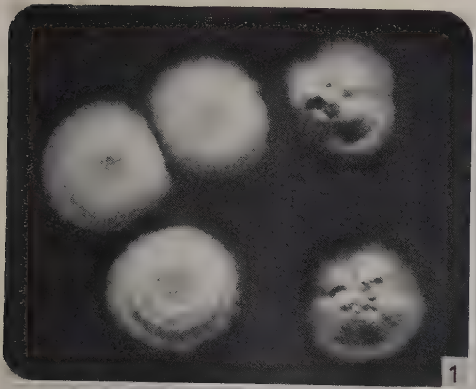
Fig. 4. A wholly *crinkled* colony (top). A sectored *waxy* colony (right), with morphologically normal sector.

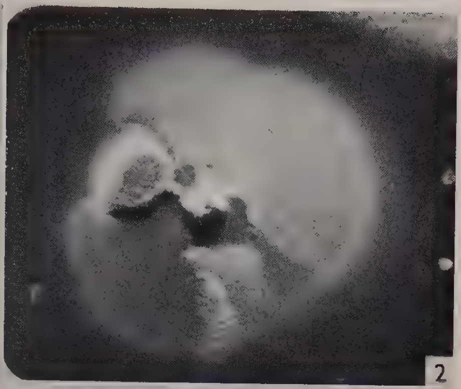
Fig. 5. Two colonies of smaller size (*minutes*) (near top). Two *crinkled* colonies with *stubble* sectors (lower left and middle right).

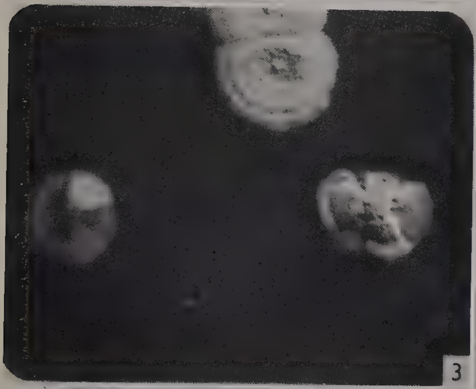
Fig. 6. Streak from spores of unstable line number 2; light areas are fluffy (morphologically normal) and dark areas *waxy*.

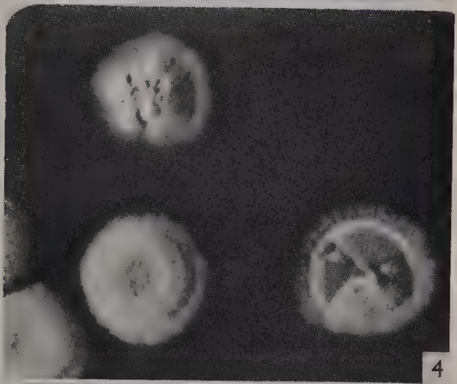
(*Received* 15 *December* 1952)

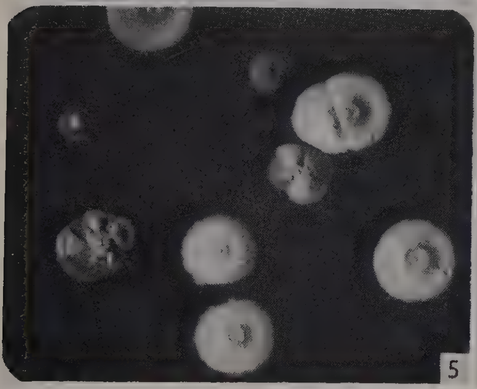
Journal of General Microbiology, Vol. 9, No. 1

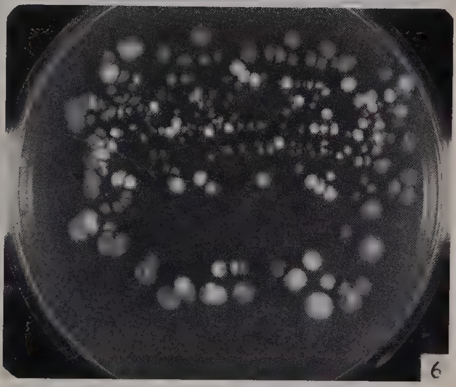

1


2


3


4


5


6

H. B. NEWCOMBE—INSTABILITIES IN STREPTOMYCES. PLATE 1

Rowley, D. (1953). *J. gen. Microbiol.* 9, 37–43.

Interrelationships between Amino-acids in the Growth of Coliform Organisms

By D. ROWLEY

The Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London, W. 2

SUMMARY: Three hundred and fifty-six strains of *Bacterium coli*, which were capable of growing on a simple salt medium containing ammonia as sole nitrogen source and glucose, were tested for inhibition of growth by added amino-acids. Inhibitions by certain amino-acids were found, and each inhibition could be prevented by the simultaneous presence of one or more other amino-acids. It was not possible to correlate these inhibitions with any other biochemical property of the strains. There was, however, a remarkably high proportion of serine-inhibited strains amongst those which had been isolated from cases of infantile gastro-enteritis.

There are numerous reports in the literature concerning the inhibitory effects of naturally occurring amino-acids on the growth of various organisms under special conditions. A classic example is the study by Gladstone (1939) of the growth of several *Bacillus anthracis* strains in defined amino-acid media; when leucine or valine separately were added in concentrations as low as 1 μg./ml. growth did not occur; when both amino-acids were simultaneously present or were both absent the organisms grew well.

Porter & Meyers (1945) described the inhibition of *Proteus morganii* growing in a chemically-defined medium, by threonine, norvaline or norleucine; several amino-acids prevented the effects of the two former but only methionine consistently prevented the norleucine inhibition. The inhibition of one strain of *Bacterium coli* by D-serine in amounts of the order of 5 μg./ml. was described by Davis & Maas (1949); this effect was wholly preventable by glycine or alanine and partly by histidine and leucine. These authors investigated some of the characteristics of the inhibition, and reported that it was mainly an increase up to 48 hr. in the lag period; bacteria which grew up after this time were just as sensitive as the originals. It was, however, possible to pick serine-resistant mutants, the proportions of which were increased by ultraviolet irradiation.

In spite of the numerous instances of microbial inhibition by amino-acids, a survey of such inhibitions within any one species has not been made. Such a survey would indicate whether these inhibitions are common to all strains of the given species and whether any differences observed might be correlated with other biochemical properties of the different strains. It has already been reported (Tatum, 1949; Rowley, 1953) that the K12 strain of *Bact. coli* when growing on the minimal medium of Davis & Mingioli (1950) was completely inhibited by as little as 0·5 μg./ml. of DL-valine, and that this effect was reversed by the simultaneous presence of leucine or isoleucine. A collection has now been made of 356 strains of *Bact. coli*, and each of these has been examined on minimal medium for the effects of various amino-acids. The same strains have been concurrently tested for sensitivity to the more common antibiotics, for lysis by the coliphages T1–T7 and for inhibition by various microbial growth factors.

METHODS

Most of the *Bact. coli* strains used were collected in this hospital from routine plates on MacConkey or deoxycholate citrate media; Dr Joan Taylor (Central Public Health Laboratories, Colindale) supplied thirty-eight strains; twelve strains were provided by Dr Studzinski of Glasgow. A small percentage of the strains would not grow on the minimal medium used (Davis & Mingioli, 1950) and were discarded. Stock cultures were kept on minimal medium agar slopes at 2°. Subcultures in liquid minimal medium were made from these parent cultures on the day of testing and grown for 8 hr. at 37°, by which time growth was just visible. Dilutions of 1 in 100 were made from these cultures, minimal medium plates were flooded with the diluted suspensions and the excess was poured off. These seeded plates were then dried for about 15 min., after which six 9 mm. holes were punched in each plate with a corkborer, to receive the amino-acid solutions.

The amino-acids used initially were all racemic mixtures as supplied by British Drug Houses Ltd.; these were made up freshly every 2 weeks in sterile water at 1 mg./ml. One drop of the required amino-acid solution was pipetted into one of the holes in the agar plates with a sterile Pasteur pipette and the procedure repeated with a fresh pipette for each of the twenty-four amino-acids, thus using four plates for each strain to be tested. After overnight incubation at 37° the plates were examined and any inhibitions greater than 18 mm. total diameter recorded.

Throughout this work all glassware and Petri dishes were cleaned with chromic + sulphuric acid mixture before being refilled with minimal medium. Without this precaution irregular results were obtained. Inhibition tests were also performed with liquid minimal medium in $3 \times \frac{1}{2}$ in. tubes, using approximately 1000 organisms/ml. as inoculum; presence of growth was read after 18 hr. at 37°. Antibiotic assays were done initially by the cup-plate method; strains which varied greatly from the average were titrated for sensitivity in $3 \times \frac{1}{2}$ in. tubes as above.

RESULTS

Of the 356 strains tested the great majority conformed to one pattern and were inhibited to varying degrees by norleucine and by norvaline. A proportion of the strains were sensitive also to other amino-acids, and the distribution of the common types is shown in Table 1, which shows the results with 326 strains. The remaining thirty strains showed multiple inhibitions of various kinds and were not easily classifiable. In Table 2 the number of strains inhibited by each amino-acid is listed; each acid is considered separately and the range of inhibitory levels of the different strains when tested in liquid minimal medium is also shown where determined.

Table 1. *Distribution of some of the more common patterns of inhibition by amino-acids among* Bacterium coli *strains*

+ = inhibition by specific amino-acid; − = no inhibition

Penicillin resistance was observed against 1000 u. penicillin/ml.

No. of strains showing given pattern	Response to presence of single amino-acid												Penicillin-resistant strains in given group (%)
	Nor-valine	Nor-leucine	Cystine	Serine	Lysine	α-Amino-butyric acid	Aspartic acid	Histidine	Tyro-sine	Methionine	Valine	Threonine	
161	+	+	−	−	−	−	−	−	−	−	−	−	22
91	−	+	−	−	−	−	−	−	−	−	−	−	21
9	+	−	−	−	−	−	−	−	−	−	−	−	100
14	−	−	−	−	−	−	−	−	−	−	−	−	40
9	+	+	−	+	−	−	−	−	−	−	−	−	22
8	+	+	+	+	−	−	−	−	−	−	−	−	29
2	+	+	−	−	+	−	−	−	−	−	−	−	.
20	+	+	+	−	−	−	−	−	−	−	−	−	25
1	+	+	−	−	−	−	+	−	−	−	−	−	.
2	+	+	−	−	−	−	−	+	−	−	−	−	.
2	+	+	−	−	−	−	−	−	−	+	−	−	.
1	+	+	−	−	−	−	−	−	−	−	−	+	.
3	+	+	−	−	−	+	−	−	−	−	−	−	.

Table 2. *Distribution of inhibition of growth by specific amino-acids among* 356 *strains of* Bacterium coli

	Norvaline	Norleucine	Cystine	Serine	Lysine	α-Amino-butyric acid	Aspartic acid	Histidine	Tyrosine	Methionine	Valine	Threonine
No. of strains inhibited by each amino-acid	261	333	40	42	3	4	13	2	2	4	3	2
Average sensitivity to amino-acids (μg./ml.)	2	1–12	0·5–2	1–15	.	.	0·5	.	.	.	0·5	.

None of these inhibitions was demonstrable on nutrient agar plates, but inhibition could be seen on minimal medium plates supplemented with 1 % of purified gelatine, casein or egg albumin. This shows that the lack of inhibition on nutrient agar must be due to the presence of reversing substances free in the medium and not to their formation during growth. Prevention of the inhibition could always be obtained; with many strains this was clear-cut and specific for one or two amino-acids. Thus the inhibition of strains 27 and 29 by norleucine was still present when all the other amino-acids, except methionine, were added at the same time. With other strains results were less definite, and several amino-acids were capable of partially preventing the inhibition. Whether this is a characteristic of those organisms or whether it is due to trace impurities in some of the amino-acids is not clear. Results of the tests of prevention of inhibition are shown in Table 3.

Table 3. *Prevention of specific amino-acid inhibitions of growth of* Bacterium coli *by other specific amino-acids*

No. of strains tested	Inhibition by	Prevented by	Partial prevention of inhibition by
1	Valine	Leucine or isoleucine	
1	Valine	Leucine or isoleucine or methionine	Glycine or asparagine
12	Norleucine	Methionine	
5	Norvaline	Methionine or leucine	
3	Lysine	Methionine	α-Amino-butyric acid or isoleucine + alanine
4	Serine	Glycine	Methionine or alanine
8	Cystine	Methionine	Asparagine + isoleucine
1	Aspartic	Lysine	
1	Aspartic	Valine	Leucine or methionine
1	Methionine	Lysine or leucine	Arginine or glutamic or tyrosine

Further experiments to throw some light on the nature of these inhibitions by specific amino-acids were largely restricted to the K12 strain. Cultures of K12 inhibited by 1–20 μg./ml. of valine grew up in the course of 48 hr. or longer and finally reached a turbidity approximately equal to that of an uninhibited control. The resulting bacteria were as sensitive to the action of valine as the original K12 organisms, demonstrating that this delayed growth was not due to the emergence of valine-resistant organisms. To decide whether the inhibition was bacteriostatic in nature, a suspension in minimal medium was made containing 50,000 K12 organisms/ml. and 10 μg. valine/ml. The number of viable bacteria was counted at 2-hourly intervals by the method of Miles & Misra (1938). For 8 hr. the count on these inhibited bacteria stayed constant, and during this time the control culture without valine had increased at least tenfold in numbers, suggesting that the inhibition is entirely bacteriostatic.

Association of these amino-acid inhibitions with other properties was looked for; in particular the strains were all examined for sensitivity to the commoner antibiotics by the same method as was used for the amino-acids. The following concentrations were used: penicillin 200 u./ml., streptomycin 100 μg./ml., aureomycin, 40 μg./ml., terramycin 40 μg./ml. and chloramphenicol 20 μg./ml. The result was read as diameter of zone of inhibition; this method was used as a first approximation in order to pick out strains which differed greatly from the average. Such strains were retested by a more accurate tube assay. The only positive correlation between these results and the inhibitions recorded in Tables 1 and 2 is that all the nine strains which were inhibited by norvaline but not by norleucine were resistant to at least 1000 u. penicillin/ml., whereas the proportions of penicillin-resistant strains in the other groups range from 20 to 40 % (see Table 1). The sensitivities to the T1–T7 coliphages bore no relation to the amino-acid inhibitions.

The strains were also tested for inhibition by several microbial growth factors, these were: *p*-aminobenzoic acid, thiamine, nicotinamide, biotin, glutathione and yeast nucleic acid (all at 1 mg./ml.). Some of the strains were greatly stimulated in growth by some of these compounds; there were very few inhibitions except with *p*-aminobenzoic acid which affected twenty of the strains, three markedly. One other strain was inhibited by yeast nucleic acid. These inhibitions were unrelated to any of the amino-acid inhibitions.

Thirty-eight strains of *Bact. coli* supplied by Dr Joan Taylor were obtained from babies with gastro-enteritis from geographically widely differing sources; many of these strains were of known antigenic structure. The results of tests on these strains are shown in Table 4. From this it can be seen that organisms of the same serological type presented the same inhibition pattern. There was, however, no universal property which might indicate reasons for the possible pathogenicity of these strains. Nevertheless, it seems remarkable that out of the thirty-four strains which grew on minimal medium as many as twenty-two (65 %) were inhibited by serine, which is a high proportion when compared with the twenty (6 %) serine-inhibited strains from 318 other coliform strains picked at random. These inhibitions were confirmed by tube assay; serine-

Table 4. *Inhibition of growth of strains of* Bacterium coli *isolated from gastro-enteritis cases, by specific amino-acids*

Antigenic type*	No. of strains tested	Inhibited by
0111 B4 H2	8	Norleucine, norvaline, serine or aspartic acid
0111 B4 H12	7	Norleucine
055 B5 H6	1	Norleucine, norvaline, serine or aspartic acid
026 B6 H-	4	Norleucine or serine
Unknown	5	Norleucine or norvaline
Unknown	9	Norleucine or serine
Unknown	4	Did not grow well in minimal medium

* Data supplied by Dr Joan Taylor.

sensitive strains were inhibited by 1–15 μg. serine/ml., whereas all the other strains grew readily in the presence of 500 μg. serine/ml. It may be significant in this connexion that Dubos (1949), examining the toxicity of 0·01 % DL-serine to strains of *Mycobacterium tuberculosis* grown on a simple medium, found that the virulent strains were most susceptible to inhibition.

DISCUSSION

Since all the organisms examined grew readily on medium lacking amino-acids, they must all possess the enzymes necessary for the synthesis of these acids from ammonium salts and glucose. Yet in the case of some strains the addition of a given amino-acid prevented or delayed growth. The presence of the amino-acid which abolished this inhibition presumably supplies an end product of the particular inhibited pathway and the organism grows freely.

From the work of Bonner (1946) and Tatum (1949), it appears that addition of valine to a culture of K12 causes an accumulation of the precursor ketovaline, and this in turn prevents the conversion of ketoisoleucine to isoleucine. When a culture 'escapes' from the inhibition caused by valine this is probably due to the accumulation of ketoisoleucine to a concentration comparable to that of the ketovaline, thus permitting growth to be resumed. Analogous to this complex interrelationship of isoleucine and valine, there may be metabolic interrelationships between all the inhibitory amino-acids and their antagonists.

These properties of amino-acid inhibitions have recently been shown to be characteristics of the organisms controlled by genetic factors (Manten & Rowley, 1953), and it seems probable that the many variations in the present collection of organisms represent a state arrived at by the natural selection of spontaneous mutants. It might, therefore, be possible to induce and select artificial mutants of these types from one single parent stock. This is now being done with some success (Clowes & Rowley, 1953), and may enable these characters to be used as additional genetic markers which do not affect the prototrophic character of recombinants.

I wish to thank Sir Alexander Fleming for his continued encouragement. I am grateful to Dr C. W. Morley of this Institute, Dr Studzinski of the Royal Hospital for Sick Children, Glasgow, and Dr Joan Taylor for collecting and sending strains to me. My thanks are due to my colleagues, Dr A. Manten and Dr R. C. Clowes, for helpful discussions; and to Mr C. R. Jenkin for his excellent technical assistance.

REFERENCES

Bonner, D. (1946). Further studies of mutant strains of *Neurospora* requiring isoleucine and valine. *J. biol. Chem.* **166**, 545.

Clowes, R. C. & Rowley, D. (1953). Selection of amino-acid inhibited mutants of *Bact. coli*. *J. gen. Microbiol.* (in the Press).

Davis, B. D. & Maas, W. K. (1949). Inhibition of *E. coli* by D-serine and the production of serine resistant mutants. *J. Amer. chem. Soc.* **71**, 1865.

Davis, B. D. & Mingioli, E. S. (1950). Mutants of *E. coli* requiring methionine or vitamin B12. *J. Bact.* **60**, 17.

DUBOS, R. (1949). Toxic effects of DL-serine on virulent human tubercle bacilli. *Amer. Rev. Tuberc.* **60**, 385.

GLADSTONE, G. P. (1939). Inter-relationships between amino-acids in the nutrition of *B. anthracis*. *Brit. J. exp. Path.* **20**, 189.

MANTEN, A. & ROWLEY, D. (1953). Genetic analysis of valine inhibition in the K12 strain of *Escherichia coli*. *J. gen. Microbiol.* **9**, x.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

PORTER, J. R. & MEYERS, F. D. (1945). Amino-acid inter-relationships in the nutrition of *Proteus morganii*. *Arch. Biochem.* **8**, 169.

ROWLEY, D. (1953). Inhibition of *E. coli* strains by amino-acids. *Nature, Lond.* **171**, 80.

TATUM, E. L. (1949). Amino-acid metabolism in mutant strains of micro-organisms. *Fed. Proc.* **8**, 511.

(*Received* 13 *January* 1953)

SUGDEN, B. (1953). *J. gen. Microbiol.* 9, 44–53.

The Cultivation and Metabolism of Oligotrich Protozoa from the Sheep's Rumen

BY BRENDA SUGDEN

The Rowett Research Institute, Bucksburn, Aberdeenshire

SUMMARY: Suspensions of the oligotrich protozoan, *Metadinium medium*, were extracted from whole rumen contents by allowing the large protozoa to settle out from the bulk of the rumen liquor and concentrating by pouring off the supernatant. The settled protozoa were separated from the associated rumen debris by incubation with maltose, when a bacterial fermentation set in with a vigorous production of gas which carried most of the rumen debris to the surface for removal by gentle suction. After repeated washing of the protozoa by resuspension in buffer and removal of the supernatant, *M. medium* was cultivated on powdered cotton-wool (0·05%) and powdered hay (0·05%) in buffer at pH 6·8. These oligotrich protozoa remained alive and dividing for 12 days. A species of *Entodinium*, concentrated from rumen liquor and washed by centrifugation, after the larger protozoa had settled out, was kept alive for 12–14 days under similar conditions in the presence of rice starch grains instead of powdered cotton-wool. Streptomycin (560 μg./ml.) was used to prepare cultures of protozoa which were bacteriologically sterile when tested by streaking agar plates containing yeast extract, starch and glucose or cellobiose. Under these conditions, *Entodinium* spp. disappeared within 24 hr. and *M. medium* in 3 or 4 days. This suggests that these organisms are dependent on bacteria for some of their metabolic processes. It is concluded that the species of *Metadinium* studied here contained symbiotic cellulolytic bacteria which were destroyed by streptomycin, but that the protozoa continued at least for some time to draw on their polysaccharide reserves after treatment with the antibiotic. Rumen oligotrichs may be of use to the sheep by acting as 'reservoirs' of polysaccharide and so preventing rapid bacterial breakdown. The relationship between the protozoa and the ruminant is considered to be a true symbiosis.

There are several records of attempts to cultivate oligotrich protozoa of the rumen contents of ruminants; the most successful attempts were those of Hungate (1942, 1943) in which he kept alive certain *Eudiplodinium* species for a year and more. Earlier attempts by Knoth (1928), Margolin (1930), and Westphal (1934) met only with very limited success. In the present work, Hungate's technique (1942) was followed quite closely but with different species of oligotrichs. The species were identified from Dogiel's monograph (1927) and Bhatia (1936; taken from Kofoid & MacLennan, 1932). Antibiotics have been widely used as a means of preparing bacteria-free protozoa but with emphasis on the preparation of cultures of flagellate protozoa. The aerobic ciliate, *Tetrahymena geleii* (now called *T. pyriformis*), survived treatment with 1000 units streptomycin/ml. (Phelps, 1947); but d'Agostino Barbaro (1951) reported that certain rumen ciliates were killed within 6 hr. by concentrations of streptomycin as low as 400 units/ml. She made no observations on the resistance of individual species.

In a previous publication Sugden & Oxford (1952) presented results on the cultivation and metabolism of holotrichs from the rumen of sheep. In the present paper investigations into the metabolism of certain species of rumen oligotrichs are reported, with experiments to find how dependent they are on bacteria for their digestive processes.

METHODS

The rumen material was obtained from two sheep, nos. 272 and 879, fed on a hay diet, and sheep 8060 fed on a diet of hay and dried grass (for the history of these animals see Oxford, 1951; Sugden & Oxford, 1952). The rumen liquor used was extracted through permanent rumen fistulae, as described previously (Oxford, 1951). After removing the larger fragments of plant material by straining through surgical gauze, the liquor was left to stand in an incubator at 38° for 1–2 hr.; the settled deposit then contained most of the larger protozoa; the small protozoa, e.g. *Entodinium* spp., remained in suspension. The mode of separation of the protozoa after this stage depended on the species required.

Preparation of suspensions of Metadinium medium

The large protozoa, including *M. medium*, usually settled out of the rumen liquor and were concentrated by pouring off the supernatant. It was not possible to use a preliminary glucose fermentation as in the preparation of holotrichs (Heald, Oxford & Sugden, 1952), since the large oligotrichs do not give a rapid response to any added substrate. When, however, the protozoa from 1000 ml. of rumen liquor were concentrated to 250 ml. and incubated with 3 g. maltose, a bacterial fermentation set in which caused a vigorous production of gas which carried most of the rumen debris to the surface. When holotrichs also were present initially it was found better to use maltose, a substrate not utilized by holotrichs (Masson & Oxford, 1951). Gentle suction was used to draw off the top layer of rumen material, and much of the supernatant liquid without disturbing the settled protozoa. The liquid drawn off was replaced by warmed buffer and the incubation continued for a further 45 min. before the procedure was repeated. Fermentation and washing were carried out in a 250 ml. conical flask fitted with a rubber stopper and bunsen valve; before replacing this stopper, the air in the top of the flask was displaced by CO_2. After a second wash with buffer, a relatively clean suspension of *M. medium* and holotrichs was left which was concentrated to 100 ml. for inoculation into four culture flasks. This inoculum contained only *M. medium* when the sample was taken from sheep 8060, the rumen of which at the time of the experiment was holotrich-free. This was the preferred inoculum, although when holotrichs were present they did not survive long on the substrates provided.

Suspensions of Entodinium *species*

Organisms of species of *Entodinium* remained in suspension with bacteria in the original rumen liquor and could only be separated from the bacteria with difficulty. By repeated centrifugation a relatively good separation was effected but this also exposed the protozoa to excessive aeration and cooling; a single centrifugation in 100 ml. tubes at 500–700 r.p.m. for 2 min. was therefore used. This gave satisfactory results in that organisms of the *Entodinium* spp. were concentrated into a small volume and many of the contaminating bacteria were removed.

Method of cultivation

For cultivation and tests of inhibition by streptomycin, 100 ml. conical flasks, fitted with rubber stoppers and bunsen valves, were used. The inorganic buffer for all washing and cultivation purposes was similar to that described by Sugden & Oxford (1952) for holotrich culture, although precautions were taken to keep it more anaerobic. The composition of the medium was (in %, w/v): NaCl, 0·5; CH_3CO_2Na, 0·15; K_2HPO_4, 0·1; KH_2PO_4, 0·03; $CaCl_2.6H_2O$, 0·01; $MgSO_4.7H_2O$, 0·01. This solution was autoclaved at 15 lb./sq.in. for 10 min., cooled to 38°, and then a mixture of 95 % nitrogen + 5 % carbon dioxide was passed through it for 5 min., by means of a sterile Pasteur pipette containing a long plug of cotton-wool. (The efficiency of this technique was tested by bubbling some of this gas mixture for 5 min. into Wright's nutrient broth; no bacteria developed after several days' incubation at 38°.) Sterile sodium sulphide to give a final concentration of 0·01 % (w/v) in the buffer was added to improve the reducing conditions (Hungate, 1950). The final pH value of the buffer was 6·8; its pH value was stable and it remained sterile for several days when kept at 38°.

The substrates, in weighed amounts suitable for addition to the cultures each day, were sterilized in $5 \times \frac{1}{2}$ cm. Durham tubes at 140° for 1 hr. For the cultivation of *M. medium* the buffer medium contained substrate, 0·05 % (w/v) powdered cotton-wool and 0·05 % (w/v) powdered hay, added daily. The powdered cotton-wool was prepared by cutting up ordinary absorbent cotton-wool with small scissors or by soaking it in conc. HCl for 48 hr. at room temperature, and washing, drying and then grinding it for 2 hr. in a ball mill (cf. Hungate, 1950). For the cultivation of *Entodinium* spp. rice starch (0·05 %, w/v) and powdered hay (0·05 %, w/v) were added.

The original inoculum for a 100 ml. culture flask containing 75 ml. of medium was 25 ml. of concentrated protozoa. To the medium was added the appropriate substrate. Before finally closing the flask, CO_2 was bubbled in to displace the small volume of air in the neck of the flask. The cultures were incubated at 38° and the buffer and substrate were replaced daily. The pH value of the cultures remained stable and rarely fell below 6·0.

Nitrogenous supplements

Trials were made with various sources of organic nitrogen; powdered hay was finally selected. Rumen liquor, lightly spun down to remove protozoa, was added at 10 % (v/v) but had no definite stimulatory effect even when freshly removed from the sheep. Rumen liquor, freed from bacteria by centrifugation and Seitz filtration, as used for holotrich cultures (Sugden & Oxford, 1952) was likewise ineffective. Various preparations of grass juice, reported by Sugden & Oxford (1952) to improve the growth of holotrichs, were tried in the presence of powdered cellulose for oligotrich cultures. In no culture did 0·1 % (v/v) or 0·5 % (v/v) grass juice (deproteinized by boiling) or 0·1 % (w/v) of the ethanol precipitate from boiled grass juice maintain *M. medium* for longer than 8 days, which period of time was easily surpassed by a supplement of powdered hay (see later).

The use of antibiotics

Streptomycin was selected from a number of antibiotics tested (which included penicillin, aureomycin and chloramphenicol) as being the only antibiotic which suppressed at least certain of the rumen bacteria without being toxic to the oligotrich protozoa. The following concentrations (mM) of streptomycin were tested: 0·1, 0·2, 0·4, 0·6, 0·8 and 1·0; 0·8 mM streptomycin (i.e. 560 μg./ml.) was finally used. 8 ml. of a 10 mM stock solution of streptomycin in sterile water were added to the culture flasks containing 100 ml. medium as required.

Tests for absence of bacteria

Tests for the absence of bacteria from the cultures were made by streaking on agar plates. The agar medium had the following composition: Difco yeast extract, 1 %; Bacto-tryptose, 1 %; K_2HPO_4, 0·1 %; agar, 2 %; starch, 0·6 %; glucose, 0·5 %. Sometimes cellobiose, 0·5 %, replaced glucose but this did not alter the results. The plates were usually incubated aerobically at 38°; incubation under anaerobic conditions did not bring up a different growth. The sheep rumen has normally a large facultatively anaerobic bacterial population (MacPherson, 1953). When tests were made for bacteria within cells of *M. medium*, deep cultures in agar medium in test-tubes were used. The agar medium, 10 ml./tube, was kept in a water-bath at 45° before inoculation. Individual protozoa were removed from the culture flasks and taken through four washes of sterile buffer before six organisms were added to each tube of agar medium; these were incubated at 38° for 48 hr.

RESULTS

Cultivation and metabolism of Metadinium medium

When clean finely powdered cellulose was added to cultures containing *M. medium*, the organisms immediately ingested large quantities of it (Pl. 1, fig. 1). There was no rapid gas and acid formation similar to that produced by rumen holotrichs in the presence of a glucose substrate (Heald & Oxford, 1953), although within 1 hr. *M. medium* stained more heavily with Lugol's iodine than it did initially. The ingested cellulose fibres were seen to disintegrate slowly within the cells, and were finally stored as starch grains, 5–10 μ. in diameter, first in the skeletal plates and then around the periphery in the ectoplasm (see later). No chemical investigation of this starch has yet been made; but from the size, shape and iodine-staining properties of the granules, it appears to be identical with that of the holotrichs, i.e. it is amylopectin-like in composition and free from amylose. A specimen is now in the hands of the Edinburgh University Chemistry Department for further study.

When the cotton-wool was not as finely powdered its breakdown to storage starch was considerably slower. The possibility that cellulose prepared by hydrochloric acid treatment was degraded and so made more readily digestible was investigated. Parallel experiments were set up to compare the behaviour of untreated absorbent cotton-wool which had simply been cut up with scissors with that of acid-treated cotton-wool which had been ground in a mortar to particles of similar size. There was no difference in the two cultures to which powdered hay was not added; both lived for 5 days only (Table 1). In the presence of powdered cotton-wool + powdered hay, these cultures of *M. medium* regularly survived for 12 days, showing frequent division up to the eighth day, but after this the numbers dwindled. It appeared that the hay acted as a source of organic nitrogen; the cells were never seen to ingest anything particulate except powdered cotton-wool. Rice and potato starch grains were also utilized by the *M. medium* organisms but were not as effective as cellulose. The results of the attempts to cultivate *M. medium*, using various nitrogenous supplements, are summarized in Table 1.

Table 1. *Results of culture trials with* Metadinium medium

Carbohydrate substrate (50 mg. per day)	Organic nitrogen supplement (per day)	Length of life of culture (days)
No substrate	0	1
Powdered cotton-wool	0	5
Hand-cut cotton-wool	0	5
Finely-powdered cotton-wool	Rumen liquor (10 ml.)	5
Finely-powdered cotton-wool	Grass juice (0·5 ml.)	8
Finely-powdered cotton-wool	Hay (50 mg.)	12
Finely-powdered cotton-wool	Hay (50 mg.) + rumen liquor (10 ml.)	10

Quantitative determination of residual cellulose by the standard Crampton & Maynard (1938) method have consistently shown a half to two-thirds utilization of substrate over 2 to 4-day periods when powdered cotton-wool was the only source of cellulose. It is not claimed that these cultures of *M. medium* were completely free from bacteria, but that the number of bacteria present was too small to account for the rapid disappearance of cellulose, which amounted to 150–200 mg. Furthermore, no source of organic nitrogen was included in the medium, and without this, thriving cultures of cellulolytic bacteria would not develop (Hungate, 1950).

Cultivation and metabolism of Entodinium *species*

Organisms of *Entodinium* spp. were kept alive for 12–14 days under conditions similar to those used for the cultivation of *M. medium*, except that the carbohydrate used was rice starch instead of cellulose. The original inoculum (Pl. 1, fig. 2) usually contained as many as four species of *Entodinium* (i.e. *E. longinucleatum* Dogiel; *E. caudatum* Stein; *E. simplex* Dogiel;

E. nanellum Dogiel), but the population changed during cultivation *in vitro*. Neither of the smaller species (*E. simplex* and *E. nanellum*) ingested starch grains, and they often died out within a few days, presumably because the original rumen bacteria on which they fed had disappeared or had been replaced by species which were not as suitable. From about the seventh day onwards only one species survived; this organism most closely resembled Dogiel's *E. longinucleatum* except that his was slightly smaller. The organism in our cultures always stained positively with Lugol's iodine both for storage and ingested starch, and continued to divide until the death of the culture. The sudden death of these cultures after 14 days may have been due to the final disappearance of some essential component of the original rumen inoculum, but this possibility appears to be discounted by the lack of effect of adding fresh protozoa-free rumen liquor daily. Another possibility is the accumulation of a toxic waste product from the protozoa. The site of breakdown of starch grains in the organisms of *Entodinium* spp. is the central endoplasmic area known as the 'gastric sac' (see Mangold, 1929). Disintegration of the starch grains takes place here, after which the soluble products are transported and stored as small polysaccharide granules in the periphery of the cell (Pl. 1, fig. 3).

Effect of streptomycin on Metadinium *and* Entodinium *spp.*

The effect of streptomycin was tested by setting up cultures as described above, with the addition of streptomycin to a final concentration of 560 μg./ml. When replacing the medium 24 hr. later, a similar quantity of streptomycin was again included. Tests by streaking on nutrient agar plates, incubated aerobically or anaerobically, showed that this concentration of streptomycin usually inhibited all the rumen bacteria which might have grown on this medium, without unduly harming the protozoa. It was considered likely that most rumen bacteria would grow on this agar medium.

The possibility was tested of streptomycin being transferred to the agar plate in sufficient quantities to inhibit the growth of bacteria in the inoculum. One loopful of 0·8 mM streptomycin in sterile buffer was streaked on half the agar plate and an active 18 hr. culture of an intestinal streptococcus known to be sensitive to streptomycin was immediately streaked on to the same places. On the other half of the plate, the streptococcus alone was streaked. After incubation at 38° for 24 hr., the streptococcus had grown equally well on both halves of the plate. Thus it is safe to assume that there is no inhibition of growth due to a 'carry-over' of streptomycin on to the plate.

Cultures of *M. medium* were easily made free from those bacteria which were capable of growth on the test medium, but unlike the holotrichs (Heald *et al.* 1952), they became fewer and less active after 3 or 4 days; the control cultures lived up to 12 days. *M. medium* did not store starch as effectively after streptomycin treatment, whether the substrate supplied was cellulose or rice starch grains. Presumably the cells drew on their carbohydrate reserves for the short time they survived after treatment. The storage 'starch' stainable with iodine certainly dwindled.

When single cells of *M. medium* were washed and incubated in deep cultures of nutrient agar, those from streptomycin-treated cultures showed no bacterial growth whether the agar medium contained glucose or cellobiose. Pl. 1, fig. 4, shows the effect of incubating agar deep cultures made from control and streptomycin-treated cultures for 48 hr. at 38°. Similar results were obtained by incubating holotrich protozoa in agar deep cultures.

After two treatments with streptomycin (560 μg./ml.), when the agar test plates showed no growth of bacteria, the *Entodinium* spp. invariably died out. This indicated that *Entodinium* spp. were in all probability dependent on the presence of bacteria. Death of the *Entodinium* spp. was not due to a direct toxic effect of streptomycin on the protozoa, because in two experiments when resistant strains of bacteria developed *E. longinucleatum* lived on for the usual 12 days.

DISCUSSION

The concentration of streptomycin (560 μg./ml.) used should have a rapidly bactericidal effect on most bacteria, although it is not possible to say with certainty, on all bacteria. This concentration is over twenty times the dose required for the rapid killing of *Bacterium coli* according to Pulvertaft (1952). Furthermore, according to Umbreit (private communication), there is as yet no certain instance of a naturally-occurring bacterium, which has never previously been in contact with streptomycin, being initially resistant to that antibiotic. In this present work, the high concentration of streptomycin was used to prevent the development of resistant strains of bacteria. Heald *et al.* (1952) showed that when thick suspensions of young cultures of *Bact. coli* and a starch-fermenting rumen streptococcus were added to washed suspensions of rumen holotrich protozoa just before the first dose of streptomycin (560 μg./ml.), the bacteria were completely killed within 24–48 hr.

According to Wasserman, Duncan, Churchill & Huffman (1952), 25 μg. streptomycin/ml. is sufficient to inhibit cellulose breakdown by mixed rumen contents, probably consisting mostly of bacteria. There are no known records of tests for the effect of streptomycin on pure cultures of rumen cellulolytic bacteria; such pure cultures are difficult to isolate (Hungate, 1950). In view of the known properties of streptomycin (see above), it is justifiable to assume that the very high concentration (560 μg./ml.) used was likely to inhibit all such rumen bacteria, as the tests by Wasserman *et al.* (1952) showed.

Rumen holotrich protozoa have been shown to be apparently unaffected by concentrations of streptomycin from 0·1 to 0·8 mM and to continue to metabolize glucose in streptomycin-treated suspensions with the same end-products as in untreated suspensions (Heald *et al.* 1952; Heald & Oxford, 1953). This suggests their independence, for their carbohydrate metabolism, of commensal bacteria. The rumen oligotrich protozoa, however, appear to be wholly or partly dependent on bacteria. Most *Entodinium* species, apart from those already observed to be bacteria-feeders, die out within 24 hr. after streptomycin treatment. This might be expected, if it were assumed that they need bacteria for some of their metabolic processes, as these small and very actively motile cells could not survive for long without renewing their carbohydrate reserve and the only source of carbohydrate provided by the medium was rice starch.

Cells of *M. medium* are able to store considerable reserves of carbohydrate, and as they continue to live for 3 or 4 days after being treated with streptomycin they may well be able to draw on these reserves without the aid of bacteria. This assumes the probability that any commensal bacteria concerned in carbohydrate metabolism, if present, would have grown on the test medium used which was rich in yeast extract, starch and glucose. The importance of bacteria in the metabolism of *M. medium*, therefore, probably appears to be in the breakdown of ingested cellulose. It seems likely from the results of agar deep cultures that streptomycin destroys bacteria within the cells as well as in the medium. Symbiotic cellulolytic bacteria, if present in the cells, might reasonably be expected to grow in deep cultures of nutrient agar which contained cellobiose, since conditions below the top few mm. are strictly anaerobic (Hungate, 1950).

The rapid toxic effect on rumen protozoa of streptomycin reported by d'Agostino Barbaro (1951) apparently contradicts the results given in the present paper; but this may be due to differences in the method of testing. It seems that she made no attempt to simulate conditions in which the protozoa would grow, as has been done in the present work, but simply incubated whole rumen contents partly diluted with distilled water, with the antibiotic, making counts at intervals.

There is no doubt that the large oligotrichs, e.g. *M. medium*, obtain their carbohydrate substrate mainly from cellulose, and that they lose the ability to utilize cellulose in the absence of bacteria. This would suggest that they rely on symbiotic cellulolytic bacteria and do not possess a cellulase of their own as reported by Hungate (1942) for other related oligotrichs. The starch-like polysaccharide which *M. medium* stores as a result of cellulose assimilation, can be extracted from the dried cultures by the method of Pucher, Leavenworth & Vickery (1948), as was the holotrich starch (Masson & Oxford, 1951). A yield of approximately 9% starch was obtained from the dried oligotrich protozoa fraction, separated from excess cellulose and bacteria by filtration through bolting silk, when a 3-day-old culture of *M. medium* with an original total population of 289,000 cells was used.

It is known that the protozoa in the rumen are not essential to stall-fed sheep because it has been shown that when the protozoa are destroyed by starvation (Ferber, 1928) or by copper sulphate treatment (Becker, 1929) the sheep continue to live normally. It has been shown in the present paper that in some of their digestive processes the protozoa are aided by symbiotic bacteria. These may be similar to the free cellulolytic bacteria which will carry on the normal metabolic processes of the sheep rumen in the absence of protozoa (Hungate, 1942).

A way in which protozoa might be of importance to the ruminant host was originally suggested by Mangold (1929). This was the suggestion that protozoa, by converting starch and sugar to storage polysaccharide, would prevent or impede rapid bacterial attack on these fodder constituents. Since 1929 it has been shown that the products of bacterial metabolism of carbohydrates in the sheep rumen are mainly acetic, propionic and butyric acids. Heald & Oxford (1953) showed that the rumen holotrich protozoa also yield these acids together with lactic acid, by fermentation of carbohydrates; but it is not yet known what are the fermentation products of the cellulose-ingesting oligotrich protozoa, either in the presence or absence of bacteria. Both oligotrich and holotrich protozoa can store enough reserve carbohydrate to survive for several days without other metabolizable carbohydrates, so that in the short time between feeds by the host ruminant, they could continue slowly to liberate carbohydrate degradation products, after the initial burst of bacterial carbohydrate metabolism had subsided.

The conclusion which can be reached is that, in accordance with previous suggestions (Mangold, 1929; Hungate, 1942), there is a true symbiotic relationship between the host animal and the protozoa. This applies to all groups of rumen protozoa and not merely to those which digest cellulose (Hungate, 1943). When considering the role of protozoa in the rumen, one must take into account the existence of a double symbiosis, involving both protozoa-ruminant and protozoa-bacteria relationships.

My sincere thanks are due to Dr A. E. Oxford for much helpful advice throughout this study.

REFERENCES

d'Agostino Barbaro, A. (1951). Sugli effetti del trattamento in vitro della fauna protozoaria del rumine degli erbivori con antibiotici e sulfamidici. *Zootec. e Vet.* **6**, 432.

Becker, E. R. (1929). Methods for rendering the rumen and reticulum of ruminants free from their normal infusorial fauna. *Proc. nat. Acad. Sci., Wash.* **15**, 435.

Bhatia, B. L. (1936). *The Fauna of British India. Protozoa-Ciliophora.* London: Taylor and Francis.

Crampton, E. W. & Maynard, L. A. (1938). The relation of cellulose and lignin content to the nutritive value of animal feeds. *J. Nutrit.* **15**, 383.

Dogiel, V. A. (1927). Monographie der Familie Ophryoscolecidae. *Arch. Protistenk.* **59**, 1.

Ferber, K. E. (1928). Die Zahl und Masse der Infusorien im Pansen und ihre Bedeutung für den Eiweissaufbau der Wiederkäuer. *Z. Tierz. ZüchtBiol.* **12**, 31.

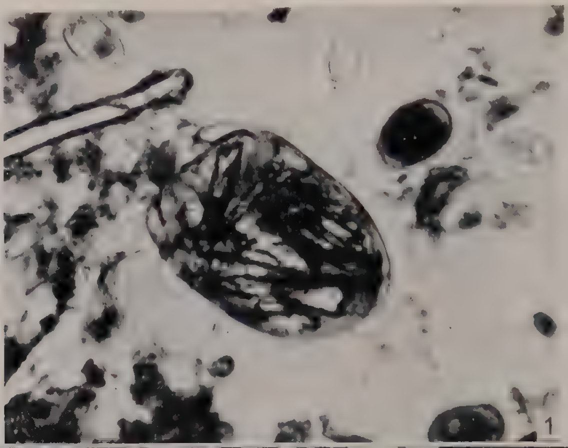
Heald, P. J., Oxford, A. E. & Sugden, B. (1952). A convenient method for preparing massive suspensions of virtually bacteria free ciliate protozoa of the genera *Isotricha* and *Dasytricha* for manometric studies. *Nature, Lond.* **169**, 1055.

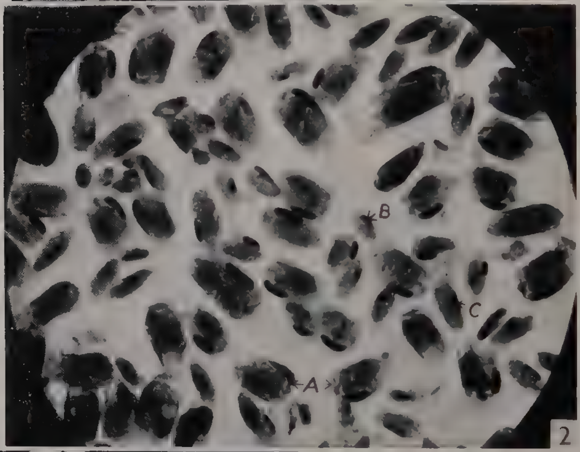
Heald, P. J. & Oxford, A. E. (1953). Fermentation of soluble sugars by anaerobic holotrich ciliate protozoa of the genera *Isotricha* and *Dasytricha*. *Biochem. J.* **53**, 506.

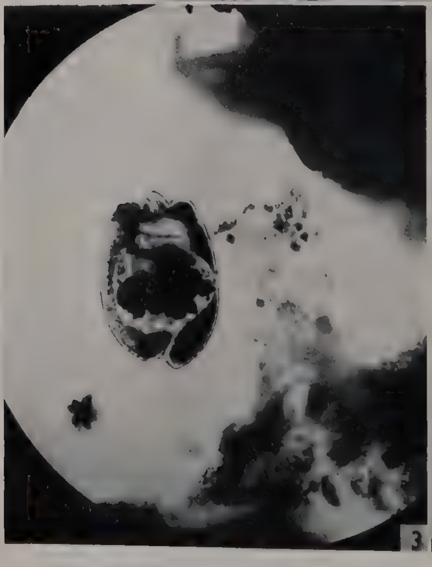
Hungate, R. E. (1942). The culture of *Eudiplodinium neglectum* with experiments on the digestion of cellulose. *Biol. Bull., Wood's Hole*, **83**, 303.

Hungate, R. E. (1943). Further experiments on cellulose digestion by protozoa in the rumen of cattle. *Biol. Bull., Wood's Hole*, **84**, 157.

Hungate, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bact. Rev.* **14**, 1.







B. Sugden—Culture and metabolism of rumen oligotrichs. Plate 1

KNOTH, M. (1928). Neue Versuche zur Züchtung der im Pansen von Wiederkäuern lebenden Ophryoscoleciden (Ciliata). *Z. Parasitenk.* **1**, 262.

KOFOID, C. A. & MACLENNAN, R. F. (1932). Ciliates from *Bos indicus* Linn. II. A revision of *Diplodinium* Schuberg. *Univ. Calif. Publ. Zool.* **37**, 53.

MACPHERSON, M. J. (1953). Isolation and identification of amylolytic streptococci from the rumen of sheep. *J. Path. Bact.* **66** (in the Press).

MANGOLD, E. (1929). *Handbuch der Ernährung und des Stoffwechsels der Landwirtschaftlichen Nutztiere. Zweiter Band*, p. 156. Berlin: Julius Springer.

MARGOLIN, S. (1930). Methods for the cultivation of cattle ciliates. *Biol. Bull., Wood's Hole*, **59**, 301.

MASSON, F. M. & OXFORD, A. E. (1951). The action of the ciliates of the sheep's rumen upon various water-soluble carbohydrates including polysaccharides. *J. gen. Microbiol.* **5**, 664.

OXFORD, A. E. (1951). The conversion of certain soluble sugars to a glucosan by holotrich ciliates in the rumen of sheep. *J. gen. Microbiol.* **5**, 83.

PHELPS, A. (1947). A method of isolating from natural waters those protozoa which can live in pure culture. *Anat. Rec.* **99**, 605.

PUCHER, G. W., LEAVENWORTH, C. S. & VICKERY, H. B. (1948). Determination of starch in plant tissues. *Anal. Chem.* **20**, 850.

PULVERTAFT, R. J. V. (1952). The effect of antibiotics on growing cultures of *Bacterium coli*. *J. Path. Bact.* **64**, 75.

SUGDEN, B. & OXFORD, A. E. (1952). Some cultural studies with holotrich ciliate protozoa of the sheep's rumen. *J. gen. Microbiol.* **7**, 145.

WASSERMAN, R. H., DUNCAN, C. W., CHURCHILL, E. S. & HUFFMAN, C. F. (1952). Effect of antibiotics on *in vitro* cellulose digestion by rumen micro-organisms. *J. Dairy Sci.* **35**, 571.

WESTPHAL, A. (1934). Studien über Ophryoscoleciden in der Kultur. *Z. Parasitenk.* **7**, 71.

EXPLANATION OF PLATE

Fig. 1. Ingestion of cellulose fibres (cotton-wool) by *Metadinium medium*. Partly crossed Nicols. × 180.

Fig. 2. Variety of *Entodinium* spp. in rumen liquor from hay-fed sheep, including *E. caudatum* (*A*), *E. simplex* (*B*) and *E. nanellum* (*C*). Haematoxylin. × 260.

Fig. 3. *Entodinium longinucleatum* digesting rice starch grains. Stained with Lugol's iodine to show ingested starch concentrated in the 'gastric sac' and storage starch around the periphery. × 450.

Fig. 4. Growth of bacteria in agar deeps after inoculation with protozoa from control (on right) and streptomycin-treated (on left) cultures. Note disruption of agar in control tubes due to gas-producing bacterial colonies. × $\frac{1}{2}$.

(*Received* 26 *January* 1953)

NEWTON, B. A. (1953). *J. gen. Microbiol.* 9, 54–64.

The Release of Soluble Constituents from Washed Cells of *Pseudomonas aeruginosa* by the action of Polymyxin

BY B. A. NEWTON
Medical Research Council Unit for Chemical Microbiology,
Biochemical Laboratory, University of Cambridge

SUMMARY: The addition of polymyxin E to washed cell suspensions of a strain of *Pseudomonas aeruginosa* caused a release of materials which had absorption maxima at 260 mμ., pentose and phosphate from the cells. This release did not occur at 2° and in this respect differed from the leakage resulting when cells were suspended in distilled water or were treated with cetyltrimethylammonium bromide (CTAB). There was an optimum concentration of polymyxin for maximum release of cell constituents, higher concentrations inhibiting the release. A similar release of cell constituents, which was prevented at low temperatures, occurred when cells were suspended in buffer at pH 5·3. Morphological changes resulting from polymyxin treatment have been studied using the electron microscope.

Polymyxin, a material with antibiotic activity produced by *Bacillus polymyxa*, was first reported in America in 1947 (Stansly, Shepherd & White, 1947; Benedict & Langlykke, 1947). In the same year workers in this country isolated an antibiotic material produced by *B. aerosporus* (Ainsworth, Brown & Brownlee, 1947). These two organisms were subsequently shown to be similar, and the antibiotic materials to be closely related. Five polymyxins produced by different strains of *B. polymyxa* have now been recognized (Brownlee & Jones, 1948); these are all basic peptides with molecular weights of about 1200. It has been suggested that they may be cyclic peptides, resembling to some extent gramicidin S (Bell *et al.* 1949). In addition to a number of amino-acids all these compounds contain a C_9 saturated fatty acid which has been identified as 6-methyl-octan-1-oic acid (Wilkinson, 1949). Polymyxin E, which was used in all the work to be described, contains the amino-acids D-leucine, L-threonine and $\alpha\gamma$-diaminobutyric acid.

There has been little work reported in the literature on the mode of action of polymyxin. Bliss, Chandler & Schoenbach (1949) observed that the antibiotic was rapidly bactericidal in low concentration against many Gram-negative bacteria, and that it was more effective at 37° than at 10°. The antibacterial activity was found to decrease with increasing size of inoculum. Some organisms, including strains of *Proteus* spp. and *Neisseria* spp., were naturally resistant, but repeated exposure to the antibiotic did not result in the development of resistance in sensitive strains. The activity of polymyxin was decreased by the presence of substances such as soap and lipositol which are known to antagonize cationic detergents. Latterade & Macheboeuf (1950) reported that polymyxin forms water-insoluble complexes with ribonucleic acids, mononucleotides, certain phospholipids and related compounds, and suggested that this may cause the agglutination of certain bacteria by polymyxin. The agglutination is inhibited by neutral salts at low concentration. Szybalski & Bryson (1952) found that certain strains of *Bacterium coli* can be made resistant to polymyxin B. Cohen, Purdy & Kushnic (1952) reported that polymyxin inhibits esterase activity in a number of mycobacteria.

Aqueous solutions of polymyxin are markedly surface active. Many surface-active agents are bactericidal, and various explanations have been put forward to account for this action. Baker, Harrison & Miller (1941) suggested that detergents disorganize the cell membrane and denature certain proteins. Hotchkiss (1946) reported the release of nitrogenous and phosphorus-containing compounds from bacteria which had been treated with detergents in bactericidal concentrations. In the case of Gram-positive organisms Gale & Taylor (1947) showed that the nitrogenous substances released included free amino-acids. Salton (1951) found purines, pyrimidines, pentose and inorganic phosphate to be released from Gram-positive and Gram-negative organisms when these were treated with cetyltrimethylammonium bromide (CTAB). Electron microscopy of cells treated with CTAB (Salton, Horne & Cosslett, 1951) showed that this detergent caused marked morphological changes. A change in surface charge of such cells was shown by McQuillen (1950), who used a micro-electrophoresis technique.

Knox, Auerbach, Zarudnaya & Spirtes (1949) showed that the enzymic activity of certain cell-free preparations of *Bact. coli* was inhibited at detergent/protein ratios which were bactericidal for the intact cell. They suggested that the specific inhibition of such detergent-sensitive enzymes could account for the metabolic inhibition, cell death and increased permeability observed in bacteria treated with bactericidal amounts of cationic detergent. However, Salton (1951) suggested that it is unnecessary to invoke the inhibition of a detergent-sensitive enzyme as being the primary cause of cell death, and concluded that there is little evidence which disagrees with the original hypothesis of Baker *et al.* (1941) that detergents act by disorganization of a cell membrane.

Since polymyxin is surface active it was decided to compare the action of this antibiotic with the cationic detergent CTAB. The organism chosen for these studies was *Pseudomonas aeruginosa* since polymyxin has been used with some success in the treatment of wounds infected with this organism (Jackson, Lowbury & Topley, 1951).

METHODS

Organism. A strain of *Ps. aeruginosa* isolated from a patient at the Birmingham Accident Hospital by Dr E. J. L. Lowbury was used in all experiments. The growth of this organism was inhibited by 5 μg. polymyxin E/ml., when an inoculum of *c.* 10^6 cells/10 ml. was used.

Medium, conditions of culture and harvesting. The organism was grown for 15 hr. at 30° in tryptic digest of casein which contained the equivalent of 3 % (w/v) casein, at an initial pH value of 7·4–7·6. Roux bottles containing 150 ml. medium were inoculated with 2 ml. of an overnight culture in the same medium. Cells were harvested by centrifugation, washed three times in 1 % (w/v) sodium chloride, and finally suspended in saline of the same strength to give a suspension containing between 12 and 15 mg. dry weight cells/ml. Dry weights were determined by drying 1·0 ml. samples of the suspension to constant weight in an air oven at 105°.

Treatment of cells. The release of cell constituents was examined after treatment of the cells in the following ways: (1) suspension in 1 % (w/v) sodium chloride; (2) suspension in distilled water; (3) suspension in 1 % (w/v) sodium chloride solutions containing polymyxin or CTAB; (4) suspensions in McIlvaine's (1921) citrate-phosphate buffers at various pH values; (5) after being pipetted into distilled water at 100° and held at that temperature for 15 min.; (6) extraction with 5 % trichloroacetic acid (TCA) at 5° for 1 hr.

In all cases the final suspension density was 1·2–1·5 mg. dry weight cells/ml.

Estimation of cell constituents released into supernatant fluid. The majority of the cells were removed from samples by centrifugation at 2000 *g* for 5 min.; the supernatant fluids were removed and clarified by centrifugation at 2000 *g* for 20 min. The ultraviolet absorption spectra of these cell-free supernatant fluids was studied by means of a Beckman spectrophotometer, corrections being made for the absorption of the suspending liquids. Total phosphate content of the supernatant fluid was determined by the method of Fiske & Subbarow (1925); pentose was estimated by the procedure of Mejbaum (1939) calibrated against D-ribose.

Electron micrographs. Washed cells suspended in 1 % (w/v) saline containing 0·03 M-phosphate adjusted to pH 7·2 were treated with various concentrations of polymyxin for 1 hr. at 30°. Micro-drops of these suspensions, suitably diluted with 1 % (w/v) saline, were placed on specimen grids covered with nitrocellulose film, fixed for 2 min. in osmic acid vapour, dried in a desiccator and then washed free of salts with distilled water. The specimens were shadowed with gold-palladium alloy (60 : 40 %). The shadowing was at an angle of 30° from the plane of the supporting film. Observations were made in the Siemens electron microscope at a direct magnification of × 10,000–14,000.

RESULTS

The leakage from washed cells of material absorbing at 260 mμ.

Examination of the ultraviolet absorption spectra of the supernatant solutions from suspensions of cells in 1·0 % (w/v) sodium chloride or distilled water showed a maximum at 260 mμ. in each case (Newton, 1953). After 3 hr. incubation at 30° the absorption of the supernatant solution from cells in distilled water was about ten times that of the supernatant solution from suspensions in saline. Addition of polymyxin or CTAB (final concentration 100 μg./ml. in each case) to the suspensions in distilled water did not result in any increase in the leakage of 260 mμ. absorbing material. These results are recorded in Table 1.

The leakage from cells in distilled water was decreased by the presence of sodium chloride. The effect of salt concentration on the leakage is shown in Fig. 1: concentrations up to 1 % (w/v) sodium chloride caused a marked

Table 1. *Release of* 260 mμ. *absorbing material from washed cells of* Pseudomonas aeruginosa

Suspensions of cells in 1 % (w/v) saline, distilled water and distilled water + 100 μg. polymyxin or CTAB/ml. The amount of 260 mμ. absorbing material released into the suspending fluid was estimated at various times during a 3 hr. incubation at 30°.

	Suspending medium			
	1 % (w/v) saline	Distilled water	Water + 100 μg. polymyxin/ml.	Water + 100 μg. CTAB/ml.
Time (min.)	Log I_0/I (at 260 mμ.)/mg. dry-weight cells			
15	0·028	0·80	0·78	0·92
30	0·043	1·80	1·64	1·91
60	0·085	2·17	2·06	2·16
120	0·190	2·40	2·23	2·42
180	0·260	2·61	2·41	2·67

decrease in the leakage; further increase in concentration had little additional effect. The addition of polymyxin or CTAB to cells suspended in 1·0 % (w/v) saline caused a rapid release of 260 mμ. absorbing material. The ultraviolet absorption spectra of the supernatant solutions after 3 hr. incubation at 30° is shown in Fig. 2.

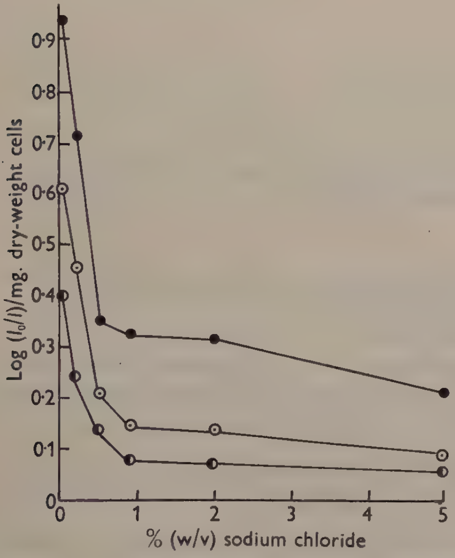


Fig. 1

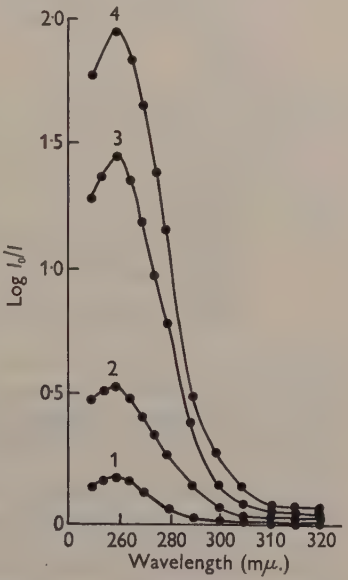


Fig. 2

Fig. 1. The effect of salt concentration on the leakage of 260 mμ. absorbing material. Washed cells of *Ps. aeruginosa* suspended in sodium chloride of varying concentrations were incubated at 30°. The absorption of the supernatant fluids at 260 mμ. was measured after 10 min. (◐—◐), 30 min. (⊙—⊙) and 180 min. (●—●).

Fig. 2. Ultraviolet spectra of supernatant fluids from *Ps. aeruginosa* suspensions (1·5 mg. dry-weight cells/ml.) in 1·0 % (w/v) saline alone or + 100 μg. polymyxin or CTAB/ml. Temperature, 30°. Curve 1, saline suspension, initial sample; curve 2, saline suspension incubated for 180 min.; curve 3, suspension in saline + CTAB incubated for 180 min.; curve 4, suspension in saline + polymyxin incubated for 180 min.

The rate of release of 260 mμ. absorbing material from suspensions of cells in distilled water, 1·0% (w/v) saline or 1% (w/v) saline + 100 μg. polymyxin/ml. was followed for 22 hr. and compared with the amount of material released by treatment at 100°. Fig. 3 shows that the release from cells in distilled water was complete in about 3 hr. Cells standing in saline released 260 mμ. absorbing material at a slow steady rate during the whole period of 22 hr. The addition of polymyxin to such suspensions resulted in an initial rapid release during the first 4–5 hr. followed by a slower release during the next 17 hr. The amount of 260 mμ. absorbing material released from polymyxin-treated cells in 22 hr. exceeded the amount which was released from the cells by treatment at 100°. Similar results were obtained when CTAB was added to cells suspended in saline.

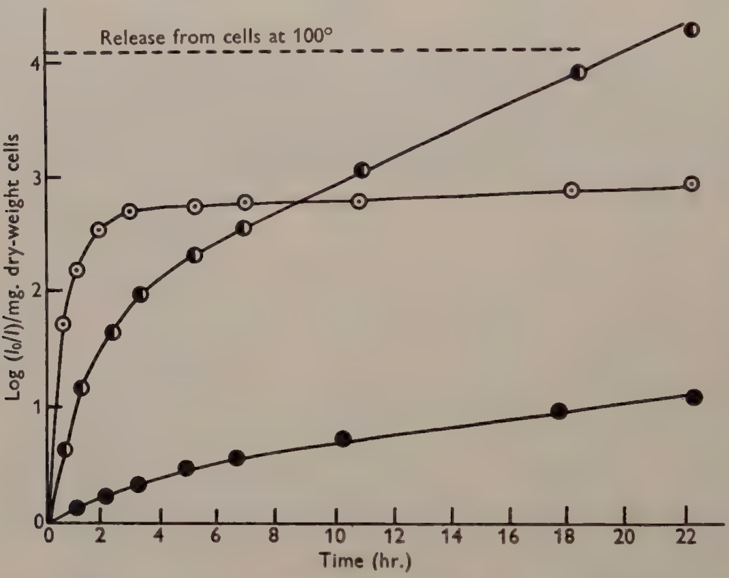


Fig. 3. Rate of release of 260 mμ. absorbing material from *Ps. aeruginosa* suspended in 1·0% (w/v) saline (●—●); distilled water (⊙—⊙); 1·0% (w/v) saline + 100 μg. polymyxin/ml. (◐—◐). Temperature, 30°.

The effect of polymyxin and CTAB concentration on the release of 260 mμ. *absorbing material*

Estimations were made of the amounts of 260 mμ. absorbing material released from cells during treatment with a range of polymyxin or CTAB concentrations from 10 to 1000 μg./ml. Samples were taken at various times during a 6 hr. incubation at 30°. Figs. 4 and 5 show that the curves obtained for polymyxin and CTAB-treated cells were essentially the same. There was an optimum concentration for maximum release of 260 mμ. absorbing material, and increase in concentration above this level caused less to be released. In the case of CTAB-treated cells, concentrations above 500 μg./ml. resulted in the immediate release of a small amount of 260 mμ. absorbing material with no further release during the next 6 hr. The amount released by these high concentrations was found to be equal to the amount extractable from the cells with cold 5 % TCA; this was only one-tenth of the amount which could be extracted from the cells by treatment at 100°. With polymyxin-treated cells similar results were obtained, but the agreement was not quite as close since the absorption of polymyxin at 260 mμ. is appreciable in concentrations above 500 μg./ml. A satisfactory method for determining the amount of polymyxin remaining in the supernatant solutions has yet to be found, and the curves are not corrected for the absorption due to this.

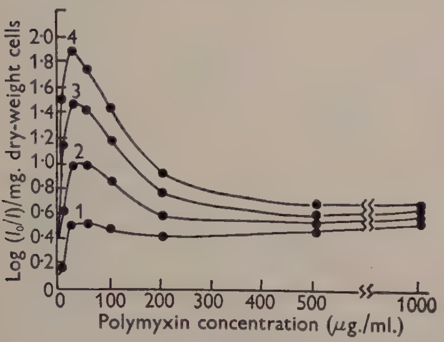


Fig. 4

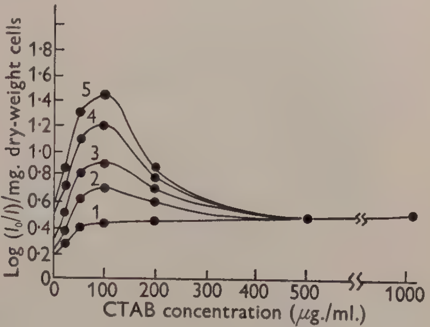


Fig. 5

Fig. 4. The effect of polymyxin concentration on the release of 260 mμ. absorbing material from *Ps. aeruginosa* at 30°. Cells were suspended in 1·0 % (w/v) saline + polymyxin and the absorption of the supernatant solution measured at 260 mμ. after the following periods of incubation: curve 1, 30 min.; curve 2, 2 hr.; curve 3, 4 hr.; curve 4, 6 hr.

Fig. 5. The effect of CTAB concentration on the release of 260 mμ. absorbing material from *Ps. aeruginosa* at 30°. Cells were suspended in 1·0 % (w/v) saline + CTAB, and the absorption of the supernatant solution measured at 260 mμ. after the following periods of incubation: curve 1, 30 min.; curve 2, 1 hr.; curve 3, 2 hr.; curve 4, 5 hr.; curve 5, 8 hr.

The release of pentose and phosphate from cells

The ultraviolet absorption of supernatant fluids from cells suspended in saline or saline + 100 μg./ml. polymyxin was measured at 260 mμ., and samples were then analysed for pentose and total phosphate content. Fig. 6 shows that the release of pentose and phosphate occurred at the same rate as the release of 260 mμ. absorbing material. Similar results were obtained with CTAB-treated cells and with cells suspended in distilled water.

The effect of pH *on the release of* 260 mμ. *absorbing material*

Washed cells were suspended in McIlvaine's (1921) citrate phosphate buffer, in the presence or absence of polymyxin, at a range of pH values between 3 and 8. Samples were taken after incubating at 30° for 1 and 3 hr., and the absorption of the cell-free supernatant solutions measured at 260 mμ. Fig. 7 shows that after 1 hr. incubation, while there was marked release at pH 5·3 there was little release from the cells suspended in buffer alone at pH values between 6·5 and 8. After 3 hr. incubation the leakage from cells in buffer alone at pH 5·3 exceeded the leakage from polymyxin-treated cells and was also greater than the amount which could be extracted from the cells by treatment at 100°.

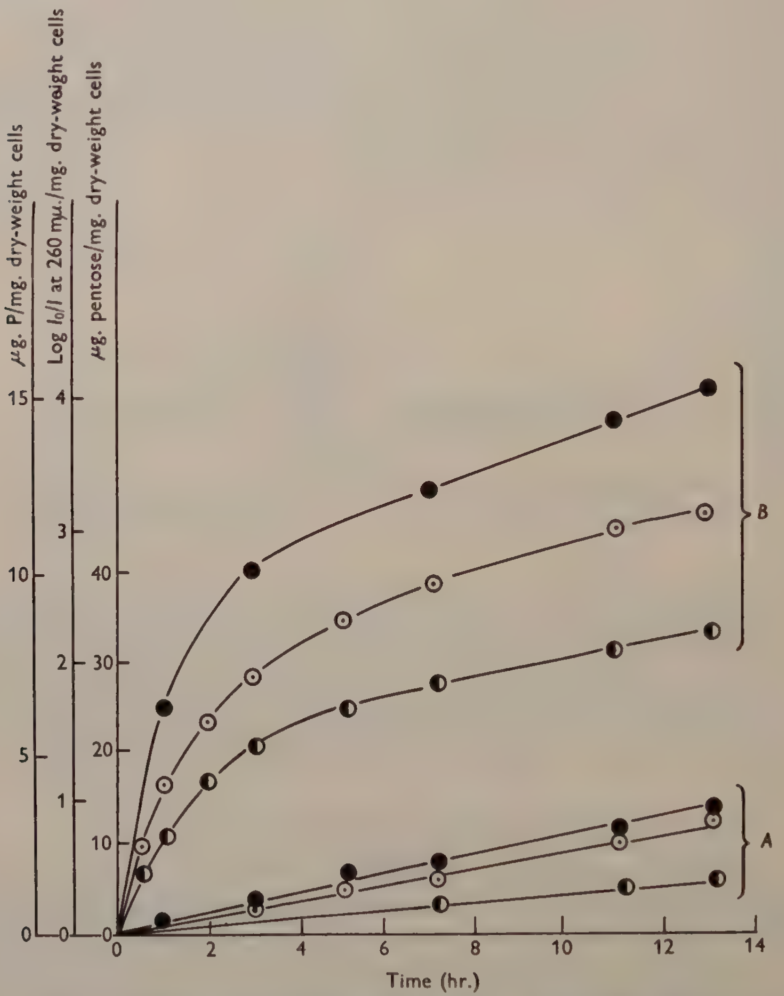


Fig. 6. Rate of release of cellular constituents from *Ps. aeruginosa* suspensions at 30°. (●—●), total phosphate; (⊙—⊙), 260 mμ. absorbing material; (◐—◐), pentose. (*A*) cells suspended in 1·0% (w/v) saline; (*B*) cells suspended in 1·0% (w/v) saline + 100 μg. polymyxin/ml.

The effect of temperature on the release of 260 mμ. *absorbing material*

It has been shown that the release of 260 mμ. absorbing materials was accelerated by suspension of cells in (1) distilled water, (2) saline + polymyxin, (3) saline + CTAB, and (4) buffer at pH 5·3. The rate of release was followed at 2 and 30° in each case, and it was found that the release was of two types: (*a*) that which occurred at both temperatures, and (*b*) that which did not occur at 2° (Figs. 8, 9). The leakage from suspensions of cells in distilled water occurred at both temperatures, whereas the leakage from cells suspended in buffer at pH 5·3 and in saline + polymyxin did not occur at 2°. In the case of CTAB-treated cells there was an initial release at 2° which was complete after 30 min., no further release occurring during the next 5 hr. The amount of 260 mμ. absorbing material released from cells by CTAB at 2° was approximately the same as that obtained by extraction of the cells with cold 5 % TCA.

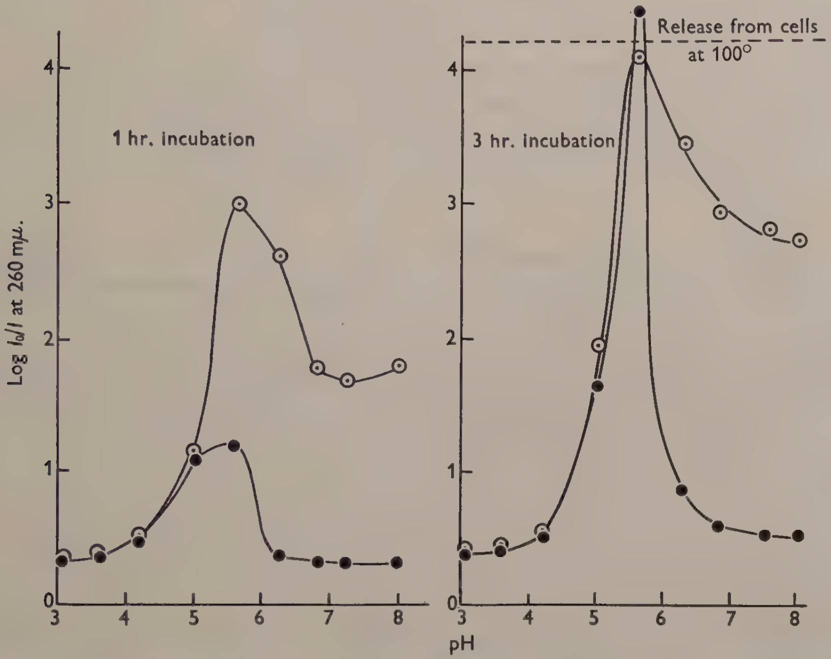


Fig. 7. Effect of pH on the release of 260 mμ. absorbing material. (●—●), cells suspended in McIlvaine's citrate phosphate buffers; (⊙—⊙), suspensions in buffer + 100 μg. polymyxin/ml.

Electron microscopical observations on polymyxin-treated cells

Electron micrographs of polymyxin-treated cells showed that the release of soluble cell constituents was accompanied by marked morphological changes. Concentrations of polymyxin which caused maximum release of 260 mμ. absorbing material also caused a maximum loss of 'electron-dense' material from the cells. Cells treated with higher concentrations of polymyxin, which inhibited the release of 260 mμ. absorbing material, retained the majority of their 'electron-dense' material although they show marked surface damage (Pl. 1, figs. 1–5).

DISCUSSION

The leakage of 260 mμ. absorbing material, pentose and phosphate which occurred when washed cells of *Ps. aeruginosa* were treated with CTAB was very different from the leakage from streptococci and staphylococci observed by Salton (1951). In the case of these Gram-positive organisms the amount of material released by the detergent equalled the amount which could be

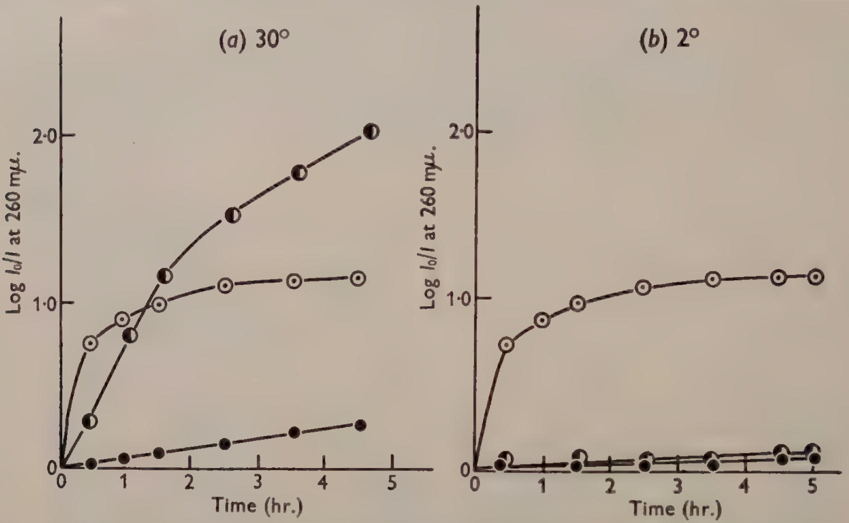


Fig. 8. Effect of temperature on release of 260 mμ. absorbing material. Cell suspensions in 1·0% (w/v) saline (●—●); distilled water (⊙—⊙); saline + 100 μg. polymyxin/ml. (◐—◐); suspensions incubated at 30 and 2°, the amount of 260 mμ. absorbing material in the supernatant fluids is plotted against time.

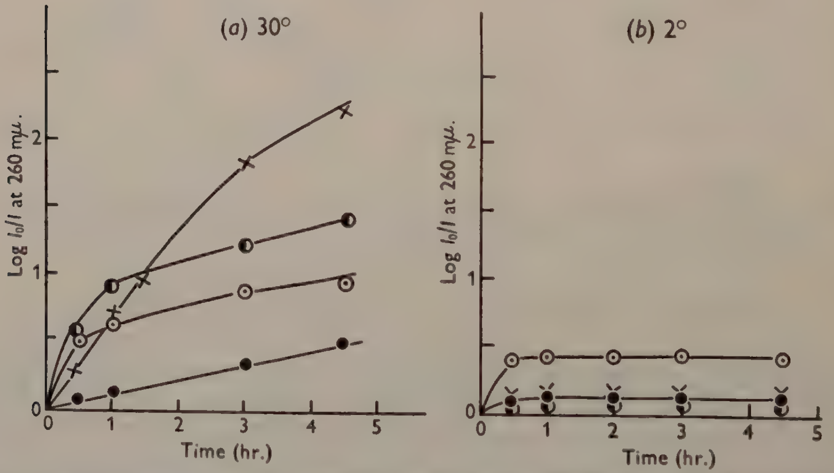


Fig. 9. Effect of temperature on release of 260 mμ. absorbing material. Cell suspensions in citrate-phosphate buffer, pH 7·3 (●—●); pH 5·3 (×—×); pH 7·3 + 100 μg. CTAB/ml. (⊙—⊙); pH 7·3 + 100 μg. polymyxin/ml. (◐—◐). Suspensions incubated at 30 and 2°, the amount of 260 mμ. absorbing material in the supernatant fluids is plotted against time.

extracted from the cells by treatment at 100°, and this release was complete in about 5 min. in the case of streptococci and 30 min. in the case of staphylococci. Increase in the concentration of detergent did not result in any marked decrease in the amount of materials released. CTAB treatment of *Ps. aeruginosa* resulted in a slow leakage which continued for many hours, and eventually exceeded the amount which could be extracted from the cells at 100°. This slow leakage did not occur at low temperatures or in high concentrations of the detergent. In each of these cases there was an initial rapid leakage of cell constituents, the amount of which corresponded very closely to the amount which could be extracted from the cells by cold 5 % TCA.

These facts could be explained by assuming that the cells contain a small amount of nucleotide, or other purine or pyrimidine-containing material which was immediately released on the addition of the detergent; this leakage could be the result of a physical disorganization of the osmotic barrier of the cells as proposed by Baker *et al.* (1941). Such an effect would not be markedly affected by temperature. The further release of cell constituents may be due to an enzymic breakdown of larger non-diffusible cell components, this breakdown being inhibited by high concentrations of CTAB or by low temperatures.

Polymyxin, in causing the leakage of 260 $m\mu$. absorbing material, pentose and phosphate from washed cells, appeared to be acting in a manner similar to CTAB. As aqueous solutions of polymyxin are markedly surface active and the molecule contains a hydrophobic chain and a polar group, and as the bactericidal activity of the antibiotic is decreased by the presence of substances known to antagonize cationic detergents (Bliss *et al.* 1949), this conclusion seems reasonable. However, the fact that the leakage produced by polymyxin did not occur at 2° suggests that it may have been entirely due to enzymic breakdown of cellular constituents, and that the antibiotic did not act primarily by altering the permeability of these cells. Alternatively, polymyxin may cause a disorganization of the osmotic barrier of the cell by chemical combination with some of its components; this combination may not occur to any significant extent at 2°. CTAB may differ from polymyxin in its action, by causing a physical disorganization which cannot be prevented by a lowering of temperature.

The author is indebted to Dr E. F. Gale for encouragement and help. He is also grateful to Dr M. R. J. Salton for samples of CTAB; to Mr H. Pearson, Mr K. Harvey and Miss E. Green, of the Cavendish Laboratory, for technical assistance in taking electron-micrographs, and to Burroughs Wellcome and Co. for a supply of polymyxin E.

REFERENCES

Ainsworth, G. C., Brown, A. M. & Brownlee, G. (1947). 'Aerosporin', an antibiotic produced by *Bacillus aerosporus* Greer. *Nature, Lond.* **160**, 263.

Baker, Z., Harrison, R. W. & Miller, B. F. (1941). Inhibition by phospholipids of the action of synthetic detergents on bacteria. *J. exp. Med.* **74**, 621.

Bell, P. H., Bone, J. F., English, J. P., Fellows, C. E., Howard, K. S., Rogers, M. M., Shepherd, R. G., Winterbottom, R., Dornbush, A. C., Kushner, S. & Subbarow, Y. (1949). Chemical studies on polymyxin: comparison with 'Aerosporin'. *Ann. N.Y. Acad. Sci.* **51**, 897.

Benedict, R. G. & Langlykke, A. F. (1947). Antibiotic activity of *Bacillus polymyxa*. *J. Bact.* **54**, 24.

Bliss, E. A., Chandler, C. A. & Schoenbach, E. B. (1949). *In vitro* studies of polymyxin. *Ann. N.Y. Acad. Sci.* **51**, 944.

Brownlee, G. & Jones, T. S. G. (1948). A related series of antibiotics derived from *B. polymyxa*. *Biochem. J.* **43**, xxv.

Cohen, S., Purdy, C. V. & Kushnic, J. B. (1952). Inhibition of bacterial esterase activity by polymyxin B. *Fed. Proc.* **11**, 463.

Fiske, C. H. & Subbarow, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375.

Gale, E. F. & Taylor, E. S. (1947). Assimilation of amino-acids by bacteria. (2) The action of tyrocidin and some detergent substances in releasing amino-acids from the internal environment of *Streptococcus faecalis*. *J. gen. Microbiol.* **1**, 77.

Hotchkiss, R. D. (1946). The nature of the bactericidal action of surface active agents. *Ann. N.Y. Acad. Sci.* **46**, 479.

Jackson, D. M., Lowbury, E. J. L. & Topley, E. (1951). *Pseudomonas pyocyanea* in burns; its role as a pathogen and the value of local polymyxin therapy. *Lancet*, ii, 137.

Knox, W. E., Auerbach, V. H., Zarudnaya, K. & Spirtes, M. (1949). The action of detergents on bacteria and bacterial enzymes. *J. Bact.* **58**, 443.

Latterade, C. & Macheboeuf, M. (1950). Recherches biochimiques sur le mode d'action de la polymyxine. *Ann. Inst. Pasteur*, **78**, 753.

McIlvaine, T. C. (1921). A buffer solution for colorimetric comparison. *J. biol. Chem.* **49**, 183.

McQuillen, K. (1950). The bacterial surface. (1) Effect of cetyltrimethylammonium bromide on the electrophoretic mobility of certain Gram-positive bacteria. *Biochim. biophys. Acta*, **5**, 463.

Mejbaum, W. (1939). Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. *Hoppe-Seyl. Z.* **258**, 117.

Newton, B. A. (1953). The action of polymyxin on *Pseudomonas pyocyanea*. *J. gen. Microbiol.* **8**, vi.

Salton, M. R. J. (1951). The adsorption of cetyltrimethylammonium bromide by bacteria, its action in releasing cellular constituents and its bactericidal effects. *J. gen. Microbiol.* **5**, 391.

Salton, M. R. J., Horne, R. W. & Cosslett, V. E. (1951). Electron microscopy of bacteria treated with cetyltrimethylammonium bromide. *J. gen. Microbiol.* **5**, 405.

Stansly, P. G., Shepherd, R. G. & White, H. J. (1947). Polymyxin: a new chemotherapeutic agent. *Johns Hopkins Hosp. Bull.* **81**, 43.

Szybalski, W. & Bryson, V. (1952). Genetic studies on microbial cross resistance to toxic agents. (1) Cross resistance of *E. coli* to 15 antibiotics. *J. Bact.* **64**, 489.

Wilkinson, S. (1949). Crystalline derivatives of the polymyxins and the identification of the fatty acid component. *Nature, Lond.* **164**, 622.

EXPLANATION OF PLATE

Fig. 1. *Pseudomonas aeruginosa*. Washed suspension of cells from a 15 hr. culture.

Fig. 2. Washed cells treated for 1 hr. with 25 μg. polymyxin/ml. About 50 % of the cells appear to have lost the majority of their 'electron dense' material.

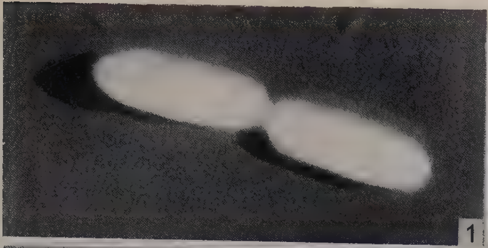
Fig. 3. Washed cells treated for 1 hr. with 50 μg. polymyxin/ml.; 98 % of the cells have lost 'electron dense' material. This concentration of polymyxin causes maximum release of 260 mμ. absorbing materials.

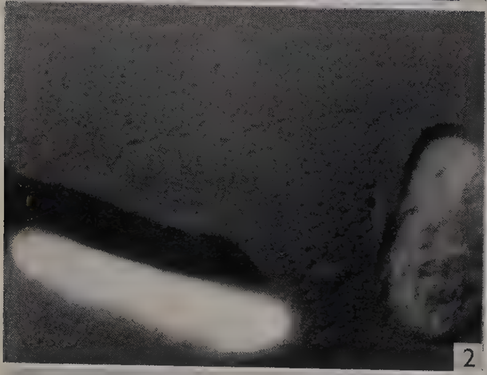
Fig. 4. Washed cells treated for 1 hr. with 500 μg. polymyxin/ml. All the cells have retained their 'electron dense' material but show some surface damage. This concentration of polymyxin inhibited the leakage of 260 mμ. absorbing materials.

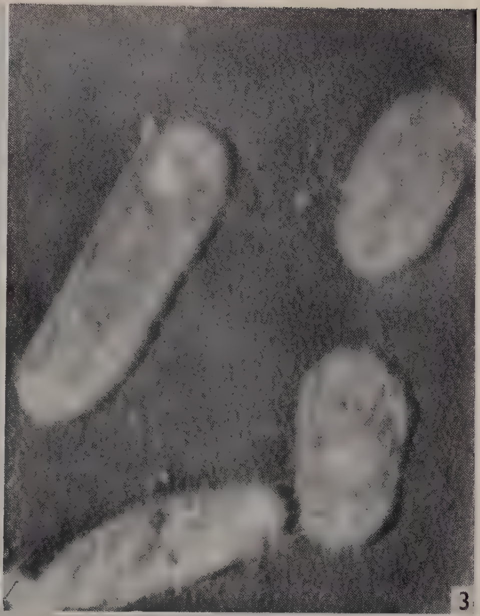
Fig. 5. Washed cells treated for 1 hr. with 1000 μg. polymyxin/ml. Cells still retain their 'electron dense' material but show marked surface damage.

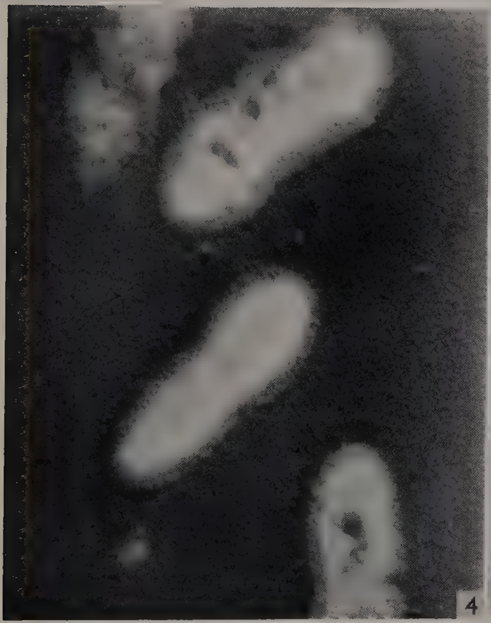
(*Received* 29 *January* 1953)

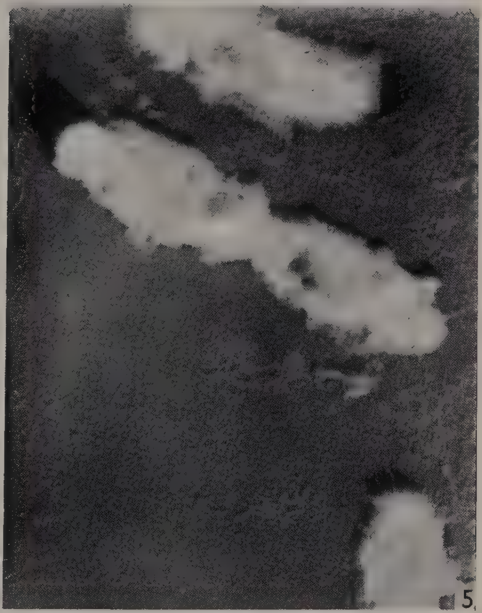
Kernforschungsanlage
Jülich
Zentralbibliothek


1


2


3


4


5

B. A. NEWTON—POLYMYXIN AND *PSEUDOMONAS AERUGINOSA*. PLATE 1

ANDERSON, E. S. & FELIX, A. (1953). *J. gen. Microbiol.* 9, 65–88.

The Vi Type-determining Phages carried by *Salmonella typhi*

BY E. S. ANDERSON AND A. FELIX

Central Enteric Reference Laboratory and Bureau, Public Health Laboratory Service (Medical Research Council), London, N.W. 9

SUMMARY: The properties of the latent phages present in twelve of the thirty recognized Vi-phage types of *Salmonella typhi* are described. The isolation and characterization of these phages presented great technical difficulties which have been partly overcome.

Nine of the latent phages were shown to control Vi-phage type specificity in the lysogenic strains that carry them. The remaining three phages have not so far been demonstrated to have type-transforming properties.

The latent phages are O phages, as indicated by their lytic action on the Vi-negative *Sal. typhi* strain O 901 and on *Sal. gallinarum.* By cross-neutralization tests the twelve phages are subdivided into four serological groups, one of which shows a considerable degree of antigenic community with an anti-O phage known to attack many salmonella species belonging to different O groups. No serological relationship was found between any of the latent phages and Vi-phage II.

The Vi-phage type of lysogenic strains of *Sal. typhi* is an expression of a resistance pattern dependent on the carried phages. These phages seem to confer resistance against unadapted Vi-phage II and simultaneously to induce phenotypic modifications in it to yield specifically adapted Vi-phage preparations.

Artificially prepared Vi-phage types of the typhoid bacillus, produced by the treatment of susceptible strains with type-determining phages, appear to be as stable as the corresponding Vi-types found in naturally occurring lysogenic strains. The fact that world-wide experience with the Vi-phage typing technique over many years has proved its epidemiological reliability suggests that the chances of the typhoid bacillus encountering type-changing phages under natural conditions are remote.

It is shown that the recently proposed method of typing bacteria by identifying the phages they carry is unsuitable for application to *Sal. typhi.*

The discovery by Craigie & Yen (1938) of a Vi phage of *Salmonella typhi* capable of specific adaptation to different strains of the organism placed in our hands a tool for the recognition of Vi-types of the typhoid bacillus that has proved, beyond any doubt, its worth in the investigation of the epidemiological problems associated with typhoid fever. It raised simultaneously questions concerning the nature of the difference between the various Vi-phage types of *Sal. typhi,* and the mechanism of adaptation of Vi-phage II to these different types of organism.

Craigie (1942, 1946) observed that many strains of *Sal. typhi* carried phages which he called 'latent' or 'γ' phages; he concluded that carriage of these phages made the host organisms untypable with Vi-phage II, and called such strains 'imperfect' Vi forms. Craigie did not make an extensive study of these latent phages. However, he was able to transform Type A into Type D1 with a phage isolated from Type D1. Felix & Anderson (1951*a*) and Anderson

(1951) demonstrated that many of the Vi-types of *Sal. typhi* were lysogenic, and that the phages they carried determined the Vi-type specificity of the strains. The action of these phages on a number of strains of *Sal. typhi* resulted in a secondary growth of lysogenic organisms of changed Vi-type. Jude, Nicolle & Ducrest (1951) showed that the phage carried by Type D6 could change Type D1 into Type D6.

Lysogenic cultures are particularly common among strains of *Sal. paratyphi B* (Nicolle, Grabar & Gibert, 1946), and Scholtens (1950) made an attempt to 'type' different strains of this organism by identifying the phages they carry. Boyd (1950, 1952) suggested this method of phage-typing for *Sal. typhi-murium* and other salmonella species. Nicolle, Hamon & Edlinger (1951) showed that type specificity in certain of the phage types of *Sal. paratyphi B*, identified according to the typing scheme suggested by Felix & Callow (1943, 1951), depended on lysogenicity, and Hamon & Nicolle (1951) carried out phage-type transformations of *Sal. paratyphi B* by treating organisms with the phages carried by heterologous types. Felix & Callow (1951) found that every strain of *Sal. paratyphi B* could be shown to be lysogenic and briefly described the 'natural' phages characteristic of each of the ten recognized phage types of the paratyphoid-B bacillus. Williams Smith (1951 *a–c*) published valuable observations on lysogenic strains in five different salmonella species.

The present paper is a detailed description of the phages isolated from various Vi-types of the typhoid bacillus.

EXPERIMENTAL

Media

'Bacto' dehydrated nutrient broth (Difco) was used as the standard nutrient basis for all media, which were prepared according to the formula of Craigie & Felix (1947) and are referred to hereafter as 'Difco' broth and 'Difco' agar; the latter contained 1·3 % New Zealand powdered agar.

Isolation of phages

During the early phases of the work the organism under test for lysogenicity was seeded lightly into 3 ml. of Difco broth and was incubated at 38·5° for periods varying from 8 to 16 hr. The culture was then centrifuged at 3000 r.p.m. for 10 min. and the supernatant was divided into two parts, one of which was heated at 57° for 40 min. while the other remained unheated. The heated and unheated supernatants were then spotted undiluted on to a lawn of an indicator strain on a Difco agar plate which was incubated at 38·5° overnight and inspected the following morning for plaques.

Higher yields of phage were obtained by growing the culture under test for lysogenicity together with the indicator strain: 100×10^6 organisms each of the test culture and the indicator strain were added to 20 ml. of pre-warmed Difco broth in a 1 oz. screw-capped bottle. The culture was incubated at 38·5° for 8 hr. and tested for phage in the way described above, with and without heating. The indicator strain employed in all primary tests was the original strain of Vi-phage Type A of *Sal. typhi* (Craigie & Yen, 1938). This strain is characterized by its susceptibility to all the Vi typing-phages and has not yet been shown to carry a phage. It was assumed, therefore, that it would probably be susceptible to most, if not all, of the phages carried by the various specific Vi-types. This assumption was fully justified, and Type A has remained the standard indicator strain in these experiments, although it has since been found that several other Vi-types, some of which are themselves lysogenic, are sensitive to many of the latent phages. However, in no instance has it been possible to show that a lysogenic type is susceptible to the phage it carries.

In each case in which a phage has been isolated from a Vi-type by growth of that type with Type A, it has also been observed that the same phage could be obtained, though in much lower titre, from the culture under test grown without the indicator strain.

Table 1 shows the twelve Vi-types of *Sal. typhi* from which latent phages have so far been isolated by the methods described, together with the designation of the phages, which is an amplification of that suggested in a former paper (Felix & Anderson, 1951*a*). Although latent phages have not yet been isolated from the remaining eighteen recognized Vi-phage types and subtypes of the typhoid bacillus, it is not unlikely that some of them carry type-determining latent phages. Most of the phages described in this paper form extremely small plaques, some no more than a few microns in diameter. They are readsorbed by the lysogenic organisms emerging as secondary growth from lysates and are sensitive to hitherto unidentified differences in solid media that may inhibit plaque formation altogether. It is possible that the latent phages not yet isolated do not show visible lysis on Type A or on any other of the known Vi-type strains of *Sal. typhi*, and that they have escaped detection because a suitable indicator strain has not yet been employed.

Table 1. *Vi-phage types of* Salmonella typhi *known to be lysogenic*

Vi-phage types	Designation of latent phages	Vi-phage types	Designation of latent phages
B3	*b*3	T	*t*
D1	*d*1	25	25′
D4	*d*4	26	26′
D6	*d*6	28	28′
F2	*f*2	29	29′
K	*k*	30*	30′

* Type 30 was recently identified by one of us (E. S. A.) but has not yet been published: its designation is provisional.

Preparation of high-titre phages

One of the most difficult problems encountered in this work was that of obtaining the latent phages in sufficiently high titre to be usable for experimental purposes, particularly as antigens to produce antisera in rabbits. While some of the phages, such as *d*1 and *d*4, could be isolated with moderate ease, none was easy to grow in titres exceeding 10^8 particles/ml., and indeed only two of the phages, namely *d*1 and *k*, have ever attained in our hands a titre exceeding 5×10^8. This difficulty was probably caused, at least in part, by the rapid production of lysogenicity in the host organisms as soon as moderately high phage titres were reached; these lysogenic organisms adsorb free phage and the titre of the latter drops. It was found that there was an optimal phage/organism ratio for each phage/indicator-strain system that would produce the maximum titre with the minimum of secondary growth of lysogenic organisms. When this ratio could be determined it was usually possible to obtain good yields of phage by adjusting the initial inocula of phage and organism accordingly. However, the ratio seemed to vary with the volume of the broth medium and the size of the container, and it was only possible to take advantage of this method to a limited extent.

A useful technique was evolved whereby the phages were allowed to establish their own optimal ratios with the host cells during series of subcultures in 20 ml. volumes of Difco broth in 25 ml. screw-capped bottles. This method was specially useful with the more difficult phages such as phages *d*6, *f*2 and 29′. As a general rule the inoculum into the first bottle consisted of 200×10^6 organisms of Type A and 1 ml. of as high-titre a preparation of the phage to be grown as was available. The actual number of particles introduced usually lay between 10^6 and 10^8. The bottle was incubated at 38·5° for 8 hr., its opacity being compared hourly with that of a control bottle inoculated with 200×10^6 organisms of Type A but no phage. At the end of 8 hr. the bottles were transferred to the refrigerator where they remained overnight. The following morning 0·1 ml. was transferred from each bottle, including the control, to 20 ml. of fresh pre-warmed Difco broth which was then incubated at 38·5° for 8 hr., the opacity of phage-containing bottles being compared hourly with that of controls as before. At the end of 8 hr. the bottles were transferred to the refrigerator. These manipulations were repeated daily, and 1 ml. was removed from each experimental bottle at the end of its 8 hr. incubation period to be titrated for phage content. All bottles were stored at 4° when their incubation was completed. With the majority of the latent phages of *Sal. typhi* the phage/organism ratio seemed to adjust itself to an optimum after a few serial transfers, so that massive lysis of the organism occurred to give a maximum yield of phage. Continuation of the transfers beyond this point resulted in a rapid increase in opacity with a drop in phage titre: this stage marked the establishment of lysogenesis in the indicator strain, and tests with the standard Vi-typing phages revealed that the Vi-phage type of the strain had been changed from Type A to a type which depended on the latent phage used. The number of serial transfers required to reach the optimal phage/organism ratio varied with the different phages. Thus, with phage *d*1, one or two transfers sufficed; with phage *t*, fifteen or sixteen were necessary.

When a culture in a transfer series showed heavy lysis, the culture preceding it in the series was subcultured to bottles containing 150 ml. of fresh Difco broth at 38·5°. The amount transferred to each 150 ml. was 0·75 ml. and a control culture of the organism alone was set up in the same way. The large volumes were incubated at 38·5° for as long as lysis continued. When it seemed that secondary growth of organisms was commencing, incubation was arrested and the preparations were sterilized by the methods listed in Table 2.

Table 2. *Methods used for eliminating the propagating strain from preparations of the latent phages of* Salmonella typhi

Heating at 57° for 40 min.	Treatment with 0·16 % toluene at 37° for 1 hr.	Filtration through Gradocol membranes (750 mμ. A.P.D.)
*d*1	*d*6	*b*3
*d*4	*f*2	*t*
.	*k*	28′
.	25′	.
.	26′	.
.	29′	.
.	30′	.

Many of the latent phages were difficult to obtain free from the organisms on which they had been grown. Phages *d*1 and *d*4 presented no trouble, as they were resistant to heat. However, all the remaining phages were heat-labile, and only a small proportion of particles survived heating at 57° for 30 min. Filtration through a Seitz EK or equivalent pad resulted in losses of up to 95 % or more of these phages; in addition, filtration of the large volumes needed was unreliable as the living bacilli penetrated the pads. Porcelain filters were not used.

The heat-labile phages are all sensitive to ether and chloroform, but some were found to be largely or completely resistant to 0·16 % toluene, a concentration which is lethal to *Sal. typhi* when acting for 1 hr. at 37°; the excess of toluene was then removed by evaporation under reduced pressure for 1½ hr. at 37°. Three of the phages were filtered through Gradocol membranes of 750 mμ. A.P.D. Table 2 shows the methods adopted for eliminating the propagating strain of *Sal. typhi* from the various phage preparations. Many of the latent phages used in the following experiments were maintained for periods of over 2 years and subjected to repeated single-plaque isolation.

Stability of the latent phages on storage at 4°

Phages *d*6, *f*2, 25′, 26′, 29′ and 30′ were found to be relatively stable on storage for 6 months at 4°. Phages *b*3 and 28′, which are sensitive to toluene and to heat, are stable at 4°. Phage *k* is sensitive to heat but not to toluene; it keeps well at 4°. Phage *t* is not only sensitive to toluene and to heat but also shows slow deterioration in the cold. Of all the phages described in this paper it is the most difficult to prepare and store.

Characteristics of plaques of the latent phages

Table 3 indicates the average size and characteristics of plaques produced by the latent phages. Drops of a suitable dilution of the phage were spotted on to a lawn of Type A of *Sal. typhi* on Difco agar plates. The plates were incubated for 7 hr. and read with a × 10 aplanat hand-lens in strong indirect lighting. The size of the smallest plaques was estimated on the basis that the limit of resolution of a ×10 lens in good lighting conditions is 10 μ. The estimation of their size is thus approximate.

Table 3. *Plaque size and characters of latent phages*

Phage	Average plaque size (mm.)	Plaque characters
*b*3	0·05–0·2	Circular with rapid secondary growth
*d*1	0·1–0·2	Circular, rapid central secondary growth
*d*4	0·1–0·2	Circular, rapid central secondary growth
*d*6	0·01–0·1	Punctate
*f*2	0·01–0·1	Punctate
k	0·01–0·1	Punctate
t	0·01–0·1	Punctate
25′	0·2	Circular, shallow, obscured after 24 hr. incubation
26′	0·05–0·15	Circular, shallow, often obscured after 24 hr. incubation
28′	0·05–0·2	Circular with rapid secondary growth
29′	0·01–0·1	Punctate
30′	0·01–0·1	Punctate

Phages *d*6, *f*2, *t*, 25′, 26′, 29′ and 30′ showed inexplicable variation in lysis on solid media, although they regularly produced lysis in broth cultures. Sometimes they would produce no plaques at all on the standard Difco agar; on other occasions all plaques would be in the neighbourhood of 10 μ. in diameter; they were rarely visible to the naked eye. This variation in lytic efficiency was apparent, not only on different batches of Difco agar, but even on occasion on different plates of the same batch.

Prolonged efforts were made to eliminate this inconstancy of lysis and to increase plaque size. Full details of these attempts will not be given as they were uniformly negative. However, as a matter of interest it may be mentioned that among the methods tried were the following: addition of various cations (Ca^{++}, Mg^{++}, Mn^{++} and Co^{++}) in a wide range of concentrations; reinforcement of the medium with a range of peptones; addition of glycerol; varying times and temperatures of incubation; varying concentrations of agar base; variations in pH value; and many others. The agar-layer method of phage titration, first introduced by Gratia (1936) and widely used of recent years in work on the T group of phages (see Adams, 1950), yielded plaques easily visible to the naked eye even with the phages giving the smallest plaques by our routine method of titration. Unfortunately, the low agar concentration employed for the superficial layer in the Gratia technique invariably resulted in the exudation of a considerable amount of heavily infected liquid which made it difficult and dangerous to handle large numbers of plates. Minute increases in the percentage of agar in the superficial layer restored plaques to their original microscopic sizes, and experiments showed that it was not possible to obtain plaques of substantially increased size in the absence of free infected liquid. It was therefore decided that the method was too unsafe for routine use in work with *Sal. typhi*.

It was found that plaques were larger when the broth culture used for preparing the bacterial lawn was diluted from the usual 1–1·5 × 10^9 organisms/ml. to 0·3–0·5 × 10^9/ml. However, as the concentration of organisms in the lawn dropped the efficiency of plating of the phages fell with it, so that the advantage of the gain in plaque size was offset by the fall in the plaque count; this led to the abandonment of the use of diluted cultures for plaque-counting experiments. It was finally observed that pouring Difco agar plates at 100° provided a medium giving the most uniformly visible and countable plaques with the phages of the micro-plaque group. Pl. 1 illustrates the plaque sizes of these phages compared with those of Vi-phage A.

Titration of the phages was carried out by spotting the various dilutions with a loop on to a bacterial lawn of the indicator strain. Six spots of each dilution were applied. A standard loop was employed with an internal diameter of 3 mm. which delivered in the hands of an experienced technician a drop of great uniformity with a volume very close to 0·01 ml. spreading to a diameter of about 1·0 cm. on the bacterial lawn. Counts were first carried out after incubation at 38·5° for 7 hr. and were checked after a further 8 hr. incubation. Each area covered by the drop, as seen through the bottom of the covered Petri dish, was divided into squares with red ink, and counts were made with a × 10 hand-lens in strong oblique lighting. An electric counter tripped by a foot-switch was employed.

Lytic spectrum of the latent phages

Table 4 shows the lytic spectrum of the latent phages on the stock strains of the recognized Vi-types and subtypes of *Sal. typhi* and on two Vi-negative control strains, namely, *Sal. typhi* O 901 and *Sal. gallinarum*. The two Vi-phage Types D 5 and H, both rare types, were omitted from Table 4, as the available cultures of these type strains were in an advanced state of 'degradation' at the time the experiments were carried out. Table 4 demonstrates that many Vi-types, some of which are themselves lysogenic, are sensitive to the latent phages. The phages have been divided into groups according to their serological reactions which will be discussed later. The corresponding twelve Vi-types carrying them are, for convenience of presentation, grouped similarly.

A number of conclusions can be drawn from the lytic reactions shown in Table 4. In the first place it is evident that several different Vi-types carry phages that are related serologically, in lytic spectrum, and, as will be shown later, in a number of other properties. This applies particularly to phages *b*3 and 28′, phages *d*1 and *d*4, and phages *d*6, *f*2, 29′ and 30′. If the characterization of latent phages were used as the method of strain identification, as suggested by Scholtens (1950), Boyd (1950) and Boyd, Parker & Mair (1951), the eight clearly defined Vi-types carrying these phages would be reduced to

Table 4. *Lytic spectrum of latent phages of* Salmonella typhi

Vi-phage type strains	Latent phages of *Sal. typhi* (tested undiluted)											
	Serological group 1		Serological group 2			Serological group 3						Serological group 4
	b3	28′	*d1*	*d4*	*k*	*d6*	*f2*	29′	30′	25′	26′	*t*
A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
B1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
B2	–	–	–	–	+++	–	–	–	–	–	–	–
B3	–*	–	–	–	+++	+	+	+	+	?	–	+
28	–	–*	–	–	–	+++	+++	+++	+++	+++	+++	–
C	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
D1	+++	+++	–*	–	+++	++	+++	+++	++	–	–	–
D4	–	–	–	–*	+++	?	+++	–	–	–	–	–
K	+++	+++	–	–	–*	+++	+++	+++	+++	–	–	+++
D2	–	–	–	–	+++	–	–	–	–	–	–	–
D6	+++	+++	+++	+++	+++	–*	–	–	–	–	+++	+++
F2	–	–	–	–	+++	–	–*	–	–	–	+++	–
29	+++	+++	+++	+++	+++	–	–	–*	–	–	+++	+++
30	+++	+++	+++	+++	+++	–	–	–	–*	–	+++	±
25	+++	+++	+++	+++	+++	–	–	–	–	–*	+++	+++
26	+++	+++	+++	+++	+++	–	–	–	–	–	–*	–
E1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–
E2	+++	+++	+++	+++	+++	–	–	–	–	–	–	±
F1	–	–	–	–	+++	++	+++	++	++	–	–	–
G	++	++	++	++	+++	?	+++	+++	+++	+++	+++	–
J	–	–	–	–	+++	+++	+++	+++	+++	++	+++	–
L1	±	±	–	–	±	+++	+++	+++	+++	(±)	–	–
L2	–	–	–	–	–	–	–	–	–	–	–	–
M	(±)	(±)	(±)	(±)	+++	?	+++	+++	?	–	–	–
N	–	–	–	–	+++	+++	+++	+++	+++	–	+++	+++
O	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
T	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–*
27	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–
Vi-negative strains												
Sal. typhi O 901	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Sal. gallinarum No. 780	+++	+++	+++	+++	+++	–	–	–	–	–	–	+++

+++ = full susceptibility to lytic action of phage; ++, +, ±, (±) = diminishing degrees of susceptibility; ? = doubtful susceptibility.
* Indicates homologous phage and lysogenic strain.

three types only, one in each group, as all strains carrying one of a group of closely related phages would appear to be identical. The importance of this will be made clear in the Discussion.

It is also apparent from Table 4 that the latent phages in any one group show closely similar lytic spectra. Exceptions to this rule are, phage *k* in group 2 and phages 25′ and 26′ in group 3. However, as will be shown later, these three phages exhibit weaker group serological relationships than do the other members of the respective groups.

Table 4 also shows that these phages can attack Vi-negative strains of *Sal. typhi*. Strain O 901, a Vi-negative variant used by one of us (A. F.) for a number of years as a control culture to detect the presence of contaminant O phages in Vi-phage preparations, is susceptible to all of the latent phages. That some of these phages can also attack salmonella types other than *Sal. typhi* is illustrated by the sensitivity of *Sal. gallinarum* No. 780 to phages *b*3, 28′, *d*1, *d*4, *k* and *t*.

Preparation of lysogenic strains

Two methods were used for the preparation of lysogenic strains. The first consisted in subcultivating the secondary growth from the phage-inoculated area resulting from the application of a drop of undiluted phage to a lawn of the indicator strain. This procedure was successful when the phage titre was sufficiently high for the lysate to yield confluent lysis; with phages producing plaques as small as those of phages *d*6, *f*2, 29′ and 30′ this required a titre of 1×10^7 or higher. For this reason the method was most successful with phages *d*1, *d*4, *k*, 25′ and 26′, all of which attained or exceeded this titre or whose plaques were large enough to cause confluent lysis in lower titres. The secondary growth was well developed after plates had been incubated for 16 hr. at 38·5°. A sweep from the centre of the phage-inoculated area was transferred to 2 ml. of Difco broth which was incubated at 38·5° until it attained an opacity of $1\text{–}1{\cdot}5 \times 10^6$ organisms. It was then tested with the routine typing phages to identify type changes and simultaneously subcultured to a Dorset egg slope for storage.

The second method was used for the phages that commonly gave titres below 1×10^7 and was essentially similar to that described earlier for the production of high-titre lysates by the transfer of 0·1 ml. inocula through a series of 20 ml. Difco broth bottles. The first bottle contained 20 ml. of Difco broth to which was added 1 ml. of the most concentrated lysate available of the phage to be used and 200×10^6 organisms in the logarithmic phase of the strain it was wished to make lysogenic. The rapidity of emergence of the lysogenic organisms varied with the phages used in exactly the same way as did the speed of production of high titre lysates, but the sequence was always the same: a phase without detectable lysis was succeeded by one of heavy lysis which gave way to a third phase of normal growth of the bacteria. The second phase marked the period of maximum destruction of susceptible organisms by the phage and the establishment of lysogenicity in the survivors; the increase of turbidity in the third phase was due to the multiplication of lysogenic organisms resistant to phage action. Each broth culture in the series was subcultured to a Dorset egg slope at the end of its 8 hr. incubation. After overnight incubation at 38·5° these slopes were tested for Vi-phage type and stored thereafter at room temperature in the dark. These subcultures reflected the successive stages in the transition of the whole culture from phage sensitivity to lysogenicity. Single-colony subcultures were also prepared of strains on which lysogenicity had been impressed, and these were tested after varying periods of storage to determine the stability of the artificially induced lysogenic state.

A simple technique was later evolved which often resulted in the production of lysogenic strains in a single manipulation, yet did not require initial phage titres higher than 10^6/ml. The procedure was as follows: a very small number of bacterial cells, between 1 and 10, was introduced into 1 ml. of a lysate containing between 10^6 and 10^7 phage particles/ml. The culture was then incubated at 38·5° for about 24 hr. The resulting culture was usually lysogenic and showed the change in Vi-phage type characteristic of the latent phage employed. It seems probable that the following sequence of events occurred: under the initial conditions of the experiment the probability of collision between a phage particle and the organisms was so small that the culture was never sterilized by the phage, even when the experiment was duplicated in a large number of tubes containing an average of 0·5 organism or less each (that is, the chances of each fertile tube containing more than one organism were low). Presumably the bacteria were able to multiply sufficiently to keep ahead of their rate of destruction by phage until the density of the culture reached a point at which a large number of organisms encountered phage particles. Most of the sensitive organisms would then be eliminated in a few cycles of phage multiplication and lysogenicity established in the survivors.

Changes in bacteriophage reactions following impressed lysogenicity

Table 5 summarizes the changes brought about artificially by treating various Vi-phage types of *Sal. typhi* with the latent phages of other type strains. Phages *b*3 and 28′ have not so far been shown to have type-transforming properties detectable by the test phages employed in this investigation. Phage *k* does not change the Vi-phage type of Type A, but makes it resistant to the polyvalent anti-O phage shown in Table 5 that is in routine use in this laboratory as a control reagent to indicate that cultures under investigation belong to the salmonella group (see Felix & Callow, 1943). Phages *b*3, 28′ and *k* have, therefore, not been included in the table.

The range of Vi-type variations outlined in Table 5 by no means exhausts the potentialities of the latent phages of *Sal. typhi*. A limited selection only of the type strains susceptible to these phages were made lysogenic. Type A was most frequently used because it was the standard indicator strain employed for detecting and propagating the latent phages. It appeared to be susceptible to all the phages carried by the different types of the typhoid bacillus. Types C, E1, O, and T have also a wide range of susceptibility to these phages.

The change of Type F1 to F2 with phage *f*2 was one of the earliest transformations carried out, and the work connected with it helped to clarify the enigma of what constituted Vi-type specificity in the lysogenic types of *Sal. typhi* (see Felix & Anderson, 1951*a*; Anderson, 1951).

Table 5. *Artificial changes in bacteriophage reactions of* Salmonella typhi

			Reactions of secondary (resistant) growth to		
			Vi-phages I and IV of Craigie & Yen (1938) (Routine test dilution [R.T.D.]=1 : 100)		Polyvalent salmonella anti-O phage (R.T.D.=1 : 30,000)
Type-transforming phage employed	Original Vi-phage type of organism treated	Vi-phage type of secondary (resistant) growth	I	IV	
*d*1	A	D1	+	−	+
	E1	Untypable Vi strain	±	−	+
*d*4	A	D1	+	−	+
*d*6	A	D6	+	±	±
	C	Untypable Vi strain	+	±	±
	D1	D6	+	−	±
	F1	F2	+	−	−
*f*2	A	29	+	±	±
	C	30	+	±	±
	D1	D6	+	−	±
	D4	Untypable Vi strain	−	−	±
	E1	New subtype of E group	±	±	±
	F1	F2	+	−	−
t	A	T	+	+	+
25′	A	25	+	−	±
26′	A	26	+	−	−
29′	A	29	+	±	±
	C	30	+	±	±
	F1	F2	+	−	−
30′	A	29	+	+	±
	C	30	+	+	±
	F1	F2	+	−	−
		Controls			
Reactions to Vi-phages I and IV and anti-O phage of Original Vi-type strains	A	.	+	+	+
	C	.	+	+	+
	D1	.	+	−	+
	D4	.	−	−	+
	E1	.	±	+	+
	F1	.	+	−	−
Naturally occurring Vi-type strains corresponding to those produced artificially	D6	.	+	±	±
	F2	.	+	−	−
	T	.	+	+	+
	25	.	+	−	−
	26	.	+	−	−
	29	.	+	±	±
	30	.	+	+	±

+ = sensitive to phage in R.T.D.; ± = partially resistant to phage in R.T.D.; − = resistant to phage in R.T.D.

There seems to be no reason why a type transformation should not be possible in every phage type of *Sal. typhi* that is sensitive to the natural phages, although many of the types so produced would not be identifiable with the existing set of adapted Vi typing-phages and would be designated as 'untypable Vi-strains' according to the more recent definition of this term (Felix & Anderson, 1951*b*).

The changes in Vi-type are often accompanied by changes in susceptibility to Vi-phages that are serologically unrelated to Vi-phage II and, more strikingly, by changes in sensitivity to the polyvalent anti-O phage referred to above. The significance of these correlated changes in phage sensitivity following the establishment of lysogenesis with a single phage will be discussed later.

It can be seen from Table 5 that as a rule the laboratory made Vi-type was identical with the corresponding naturally occurring type, but certain differences occurred to which attention should be drawn:

(1) Phage 30′ acting on Type A produced Type 29, but this differed from the naturally occurring Type 29 and that prepared from Type A with phage 29′ in being fully sensitive to Vi-phage IV.

(2) Conversely, Type 30 prepared from Type C with phage 29′ differed from the natural Type 30 and that prepared from Type C with phage 30′ in being partially resistant to Vi-phage IV.

In this case two latent phages, 29′ and 30′, acting on the same strains produce Vi-phage types that are identical except for sensitivity to Vi-phage IV. As will be shown later, the relationship between phages 29′ and 30′ is so close that this slight difference in the phage-sensitivity pattern of the lysogenic resistant growths they produce is, so far, the only means of distinguishing between them.

The stability of artificially prepared Vi-phage types of Salmonella typhi

Hamon & Nicolle (1951) prepared artificially a number of phage types of *Sal. paratyphi B* by methods similar to those described here. They demonstrated, however, that the impressed lysogenicity which controlled the phage type was less stable than that occurring naturally. In the present work, a large number of strains of *Sal. typhi* which were made lysogenic artificially was re-examined after storage for about 18 months or more, and no evidence was found that the laboratory made lysogenicity was any less stable than that occurring naturally. The Vi phage reactions of the cultures remained unchanged, and the phages that had been grafted on to the organisms could be grown from them. The following examples may serve to illustrate this point.

Table 5 shows that Type D1 is converted into Type D6 by phage *f*2. Type D1 normally carries a heat-stable phage, whereas phage *f*2 is thermolabile. A phage preparation was grown from a fresh single-colony culture of the artificial Type D6 about 20 months after its transformation, and was divided into two portions, one of which was heated at 60° for 40 min., while the other was treated with toluene. Both portions were titrated on Type A, single plaques cut and the resulting phages identified. The heated portion contained only phage *d*1 but the toluene-treated fraction contained both phage *d*1 and phage *f*2. The organism had thus been made doubly lysogenic and this double lysogenicity had remained stable for 20 months. The stability of the acquired lysogenicity has also been demonstrated by tests of large numbers of single-colony cultures.

On the other hand, it has been pointed out in previous papers (Felix & Anderson, 1951*a*; Anderson, 1951) that the state of lysogenicity in naturally occurring strains of Type F2 is unstable, and that Dorset egg cultures of Type F2 stored for long periods in the dark at room temperature throw off phage-free organisms, which belong to Type F1. The same phenomenon was found in cultures of Type F2 prepared artificially from Type F1 with phage *f*2.

The evidence at present available, therefore, suggests that the state of lysogenicity produced artificially by treating the typhoid bacillus with type-determining phages is as permanent as that found in naturally occurring lysogenic strains; and indeed, it seems probable that there is no difference between naturally occurring and artificially produced lysogenicity.

SEROLOGICAL REACTIONS

Preparation of antiphage sera

Antisera were prepared against these phages at first by intraperitoneal and later by intravenous inoculation. Intraperitoneal inoculation was employed because it was feared that lysates containing typhoid antigens might be too toxic when injected intravenously into rabbits. Dosage commenced at 2·5 ml. of undiluted lysate, followed by 5·0 ml. after a 3-day interval; subsequent doses were 10, 20, 30, 30 and 30 ml. at alternating intervals of 3 and 4 days, to a total dosage of about 130 ml. of undiluted lysate. The rabbits were bled 5 days after the last inoculation and again 2 days later.

Intravenous inoculation commenced with 2·0 ml. of 1/10 phage dilution, followed by 2·0 ml. of 1/5, 1/2·5 and 1/1·25 dilutions. These doses were followed by 2, 3, 4, 4, 5, 5 and 5 ml. doses successively of undiluted lysate at alternating 3 and 4 days intervals. The rabbits were bled 5 and 7 days after the last inoculation. No toxic reactions occurred in the rabbits inoculated intravenously.

A preliminary bleeding was carried out on all rabbits before immunization with phages. The resulting sera (I bleedings) were always tested in parallel with the post-inoculation sera (II and III bleedings) when the latter were first titrated. When subsequent tests were carried out with an antiserum of low titre a low dilution of the I bleeding from the same rabbit was always included in the series in order to exclude non-specific neutralization of the phages.

Neutralization tests

The phages and homologous antisera were mixed, each in 0·5 ml. quantities, in appropriate dilutions made up with Difco broth. Controls were included of the corresponding I bleedings and of Difco broth, each mixed with the same amount of phage. The dilution of phage used throughout was that which yielded roughly 50 to 100 plaques after being mixed with an equal volume of Difco broth. The mixtures of phage and antisera were incubated at 38·5° for 3 hr. and then kept in the refrigerator overnight. The following morning they were spotted with a standard loop delivering a volume of 0·01 ml. on to a bacterial lawn of the indicator strain, and plaque counts were made after 7 and 15 hr. incubation. Once an orientating titration of this type had been performed, more precise estimates of the serum titres could be arrived at by more closely spaced titrations between selected dilutions. The titre of an antiphage serum was arbitrarily fixed as being the dilution that neutralized about 90 % of the homologous phage particles under the conditions of the experiment.

As the result of these preliminary titrations it was possible to select suitable dilutions of the antisera for cross-tests with heterologous phages. A first cross-test was set up using only two dilutions of the sera, one equal to their titres with the homologous phages, the other up to ten times as strong as this. The phages were also used in two dilutions in this test, one yielding about 50 to 100 plaques on the indicator strain, the other in ten times this concentration. The orientating cross-tests gave an indication of the serological relationships between the various phages, and further tests were carried out with selected groups of phages using a wider range of serum dilutions. Tables 6A, 6B and 7 are examples of cross-neutralization tests showing complete serological identity between some phages and less close relationships between others.

Tables 6A and 6B, which are to be read in conjunction, show that an antiserum against phage *d*1 or phage *d*4 is able to neutralize either phage to an equal titre. Overlapping cross-reactions of varying degree occur between phages *d*1 and *d*4 on the one hand, and *k* and anti-O no. 2 on the other. The extent of overlapping appears to be greater in the sera of rabbits immunized by the intraperitoneal route than by the intravenous route. In addition, there are variations in the degree of overlapping in the sera of individual rabbits immunized by either route. Of the four sera shown in Table 6B, those against phages *d*1 and anti-O no. 2 were prepared by intraperitoneal inoculation and anti-*d*4 and anti-*k* by intravenous inoculation.

The experiments summarized in Table 7 represent the best results obtained with a group of phages (group 3) that presented great technical difficulties to which reference has been made earlier. Because of their minute plaque sizes and variability of lysis, frequent failures were experienced in neutralization tests: on a number of occasions no plaques could be seen even in the control areas free from antiserum, although preliminary titrations carried out a day or two earlier had yielded countable plaques. After many disappointments it was finally decided to abandon a strictly quantitative examination of the serological relationships between these phages, and to carry out the cross-tests with undiluted phages or low dilutions of them. Because of the conditions of performing the experiments, therefore, counts could be made only when strong neutralization occurred. Nevertheless, the results were clear-cut, in spite of the fact that the figures indicating overlapping reactions are approximate. Phages *d*6, *f*2, 29′ and 30′ are serologically identical, and are closely related to phage 25′. Phage 26′ is less closely related to phages *d*6, *f*2, 29′ and 30′ than is phage 25′; and phages 25′ and 26′ have a close serological affinity. The phages in group 3 were mostly poor antigens giving antisera with titres often

Table 6A. *Example of cross-neutralization test with antiphage sera and latent phages of* Salmonella typhi

Number of plaques produced on Type A by 0·01 ml. of phage-antiserum mixture

	Dilutions of antiphage sera													
	Anti-*d*1							Anti-*d*4						
	I	II						I	II					
Phages	100	100	200	1000	3000	12T	50T	100	100	200	1000	3000	12T	50T
*d*1	80	0	0	0	1	* 42	44	95	0	0	0	0	0	* 21
*d*4	24	0	0	0	1	* 11	25	53	0	0	0	0	0	* 7
k	118	0	0	* 37	68	110	124	107	0	*12	123	114	130	112
Anti-O no. 2	60	2	*5	25	56	58	59	66	*10	25	44	32	67	50

	Dilutions of antiphage sera														
	Anti-*k*								Antiphage anti-O no. 2						
	I	II							I	II					
Phages	100	100	200	1000	3000	12T	50T	100T	100	100	200	1000	3000	12T	Control, phage dilution in broth
*d*1	81	0	0	* 16	28	42	50	65	71	2	*9	21	35	70	72
*d*4	29	0	0	*1	6	9	17	30	38	0	*2	9	15	20	32
k	139	0	0	0	0	0	0	*12	110	0	*17	70	108	121	114
Anti-O no. 2	60	0	*5	16	45	58	49	55	50	0	0	0	*4	41	63

Notes: I = serum drawn before immunization; II = serum drawn 5 days after the last inoculation. 12T = 12,000.
* = approximate position of 'titre', indicating about 90 % neutralization (read in conjunction with Table 6B).
Phage anti-O no. 2 = one of the anti-O phages isolated during paratyphoid-B outbreaks in 1941 (Felix & Callow, 1943).

Table 6B. *Comparative strength of the four homologous and heterologous neutralizing sera recorded in Table* 6A

		Antiphage sera			
		*d*1	*d*4	*k*	Anti-O no. 2
Phages	*d*1	100	100	0·5	7
	*d*4	100	100	1·0	7
	k	10	1	100	7
	Anti-O no. 2	4	0·5	0·4	100

Note: the figure 100 indicates the strength of neutralization of a phage by the homologous antiserum, determined from the 'titre' as defined in the text.

Table 7. *Approximate percentage neutralization of the six latent phages of group* 3 *by homologous and heterologous antisera*

		Antiphage sera					
		*d*6	*f*2	29′	30′	25′	26′
Phages	*d*6	100	100	100	100	90	66
	*f*2	100	100	100	100	90	66
	29′	100	100	100	100	20	33
	30′	100	100	100	100	66	33
	25′	50	10	100	20	100	50
	26′	10	10	33	10	100	100

as low as 1 : 20, but the cross-neutralization tests were adequately controlled by low dilutions of the corresponding I bleedings.

These tests made it possible to separate the latent phages of *Sal. typhi* into the serological groups shown in Table 8. In accordance with the analysis of the experiments summarized in Tables 6A, 6B and 7 serological groups 2 and 3 were each divided into subgroups (*a*) and (*b*). In each case subgroup (*a*) comprises the phages that are serologically identical, while subgroup (*b*) contains those less closely related to subgroup (*a*).

Table 8. *Serological groups of latent phages of* Salmonella typhi

Group 1	Group 2	Group 3	Group 4
Phage *b*3 Phage 28′	(*a*) Phage *d*1 Phage *d*4	(*a*) Phage *d*6 Phage *f*2 Phage 29′ Phage 30′	Phage *t*
	(*b*) Phage *k*	(*b*) Phage 25′ Phage 26′	
	[Group 2 is related to Salmonella phage anti-O no. 2]		

The fact that eight of the nine latent phages so far shown to have Vi type-determining properties belong to two serological groups, one of which contains six of the phages, is of considerable interest. Moreover, these serological relationships are usually associated with similarities in the size and characters of plaques, resistance to heat and chemical agents, efficiency as antigens in rabbits and the Vi type-determining properties of the phages. These points will be referred to again in the Discussion.

DISCUSSION

The discovery that strains of *Sal. typhi* could be distinguished from each other by adapted preparations of Vi-phage II (the routine typing phages of Craigie & Yen, 1938) aroused a considerable amount of conjecture as to what determined the differences between the various Vi-phage types. No demonstrable antigenic differences exist between them (Felix, 1941), nor has it been possible to show differences in virulence (Felix & Anderson, 1951*b*). The first observations of importance in this field were made by Craigie (1942, 1946), who isolated from Type D1 a phage which he called a 'latent phage' or 'γ agent'. He reported that this phage transformed Types A and E1 into Type D1.

The significance of Craigie's observations does not seem to have been recognized, and this work was not followed up. The next advance resulted from the demonstration that Type F1 appeared in old Dorset egg cultures of Type F2 stored for long periods in the dark at room temperature, and that, in logarithmically growing mixtures of Types F1 and F2, Type F1 disappeared (Anderson, 1951). This phenomenon was found to be caused by a phage which was named phage *f*2; this phage could lyse Type F1 and could also convert it into Type F2 with the simultaneous production of lysogenicity. In old cultures of Type F2 some of the cells lost the phage and became Type F1. These findings and parallel features observed in a number of other Vi-types of the typhoid bacillus were briefly reported in an earlier publication (Felix & Anderson, 1951*a*). Observations similar to our own have been published by Nicolle and his colleagues with the paratyphoid-B Vi-phage types (see Nicolle *et al.* 1951; Hamon & Nicolle, 1951), and with Type D1 of *Sal. typhi* which Jude *et al.* (1951) were able to change to Type D6 with phage *d*6.

It is apparent that the determination of Vi-phage type is often dependent on lysogenicity. Moreover, the number of types that can be defined by these phages, which we have designated 'latent' phages in accordance with Craigie (1946), is larger than the number of phages so far isolated. Thus, nine phages have so far been implicated in the type determination of *Sal. typhi* strains, but some have been shown to be capable of producing several Vi-phage types, the type evolved depending on the original Vi-phage type of the strain used as substrate for the phage. For example, phage *f*2, acting on Types A, C, D1, D4, E1 and F1, will produce Types 29, 30, D6, an untypable Vi strain, a new subtype of the E group and Type F2 respectively (see Table 5). The resulting cultures of changed Vi-type become lysogenic and phage *f*2 can easily be regained from them. As far as can be determined most of the artificially prepared types, with a few exceptions some of which have been discussed on p. 76, are identical with those occurring naturally and, up to the present, seem to be quite stable.

Vi-phage type specificity

The demonstration of these type-changing phages has thrown some light on the nature of the differences in the typhoid bacillus that distinguish the various Vi-phage types and the mechanism of the adaptation of Vi-phage II to the different Vi-phage types. It can be assumed that lysogenicity is responsible for the differences between at least nine of the thirty known phage types, and, as has been pointed out previously (Felix & Anderson, 1951*a*; Anderson & Felix, 1952), there can be little doubt that in these types Vi-phage type specificity is an expression of a resistance pattern resulting from the achievement of the state of lysogenicity. It seems probable that the whole range of Vi-typing phages are phenotypic modifications of Vi-phage II (Anderson & Felix, 1952, 1953). This modification is sufficiently profound to make different adapted preparations, when used in the routine test dilutions, behave in a lytic sense towards different Vi-types of *Sal. typhi* as if they were distinct phages. However, all Vi-types of the typhoid bacillus will adsorb all the adapted Vi-typing preparations irrespective of the type specificity of the latter, lysis only taking place when the Vi-phage type of the organism and the specificity of the phage coincide (Craigie, 1940). It can therefore be assumed that the resistance to certain Vi-typing preparations that results from the acquisition of lysogenicity and defines the change in Vi-type is caused by a block in the multiplication of heterologous adapted Vi-phage II, and that this block is at some stage in the multiplication of Vi-phage II subsequent to adsorption. As the type-determining phages are O phages and the changes in Vi-type are accompanied also by changes in sensitivity to other Vi-phages than Vi-phage II, and, more significantly, by changes in sensitivity to salmonella anti-O phages, it seems probable that Vi-type specificity is a property that does not reside in the Vi antigen, although the latter is necessary for the initial attachment of Vi-phage II to the organisms. This has been discussed in a previous paper (Anderson & Felix, 1953).

We have not been able so far to isolate phages from eighteen Vi-types, and the phages carried by three of the lysogenic types (B3, 28 and K) do not appear to have Vi type-determining properties. Bearing in mind the difficulties encountered in the isolation of a number of the type-determining phages, it is possible that types that have not yet yielded such phages may do so when an effective technique has been evolved for their demonstration. On the other hand, there may also be other, as yet unidentified, processes in the bacterial cell, such as mutation, causing evolution of phage resistance and thus controlling Vi-type specificity.

One can speculate as to the nature of the change in Type A that results in the evolution of a different Vi-type after the production of lysogenicity. If it can be assumed that the focus that determines Vi-type specificity in the bacterial cell and which may be called the 'type-determinant receptor' must fit a complementary pattern in the phage before the latter can complete its cycle of multiplication, it must be concluded that this focus has a sufficiently complex pattern in Type A to enable any adapted Vi-typing phage preparation to fit it. The impressing of lysogenicity on Type A with one of the type-determining phages results in a modification of the pattern, perhaps by obscuring certain groups, so that only one or a few adapted preparations can now fit the pattern sufficiently closely to continue their multiplication. When lysogenicity is lost the occluded groups are re-exposed and the culture becomes Type A once more. This explains at least some of the phenomena previously referred to under the terms 'degradation' and 'degraded Vi strains' (Craigie & Felix, 1947; Felix & Anderson, 1951*b*; Anderson & Felix, 1953). On this hypothesis it would be expected that the change from a lysogenic specific type to Type A would occur in a single step coinciding with the loss of the type-determining phage. An observation on a strain of Type D6, isolated in November 1951, from a case of typhoid fever, has recently shown that this is so: a subculture of this strain stored at room temperature in the dark for about 12 months was found to have thrown off phage-free variants giving the reactions of Type A. These variants were easily re-converted into Type D6 by the phage carried by the parent strain.

It cannot be inferred that the receptor defining Vi-type in phage A has a pattern as multipotent in properties as that of the corresponding type-determinant receptor in Type A of the typhoid bacillus, otherwise phage A would be able to attack all Vi-types of the organism to the same titre instead of being, as it is, highly specific for Type A. It is probable, therefore, that the specific receptor of phage A fits only a restricted portion of the multipotent type-determinant receptor zone of Type A, and that the corresponding specific receptors in all the adapted Vi-typing preparations are modifications of it.

Serological relationships and other properties of latent phages

No serological relationship was found between any of the latent phages and Vi-phage II. The majority of the type-determining phages belong to one serological group (group 3) comprising two subgroups. The six phages in this group share a number of other properties, although they are responsible for the specificity of different Vi-types. Thus, they are mostly poor antigens, are heat-labile and form micro-plaques on solid media; they are all stable to treatment with 0·16% toluene. Phages *d*6, *f*2, 29′ and 30′, which by the technique so far employed are serologically indistinguishable, are more closely related to each other than to phages 25′ and 26′ in that the former induce in Type A, and in other types, resistance patterns exhibiting striking similarities. This is illustrated in Table 9.

It can be seen from Table 9 that Type A is fully susceptible to all the Vi-typing phages used in the test. Type A made lysogenic with phage *d*6 is resistant to phage A in a concentration 20 times that used for routine phage typing. There are residual sensitivities left to the adapted Vi-phages F2, 29 and 30—a feature sometimes found in naturally occurring Type D6—but sensitivity to all the other Vi-typing phage preparations, which are not included in the table for the sake of brevity, has largely or completely disappeared. Sensitivity to Vi-phage I is unchanged but the organism has become resistant to Vi-phage IV and to the polyvalent anti-O phage. The phage-sensitivity spectrum of Type A, after treatment with phages *f*2 and 29′, is identical and the organism, in fact, has become Type 29. On the other hand, Type A made lysogenic with phage 30′ becomes Type 29 with the difference that this artificially prepared type is fully sensitive to Vi-phage IV, whereas the natural Type 29 and those prepared artificially from Type A with phages *f*2 and 29′ are partially resistant to Vi-phage IV (see also Table 5).

Table 9. *Production of lysogenicity in Vi-phage Type A with phages d*6, *f*2, 29′ *and* 30′

	Reactions of resultant cultures to routine test dilutions of							
	Adapted Vi-typing phages					Unadapted Vi-phages		Poly-valent anti-O phage
	A	D6	F2	29	30	I	IV	
Cultures	1500*	10000	5000	20000	10000	100	100	30000
Type A	CL	CL	CL	CL	CL	CL	CL	CL
$\frac{\text{A}}{\text{Phage } d6}$	—	CL	*μ*	*μ*	*μ*	CL	*μ*	*μ*
$\frac{\text{A}}{\text{Phage } f2}$	±*s*	CL	CL	CL	CL	CL	*μ*	*μ*
$\frac{\text{A}}{\text{Phage } 29'}$	±*s*	CL	CL	CL	CL	CL	*μ*	*μ*
$\frac{\text{A}}{\text{Phage } 30'}$	±*s*	CL	CL	CL	CL	CL	CL	*μ*

* = phage A was used in a concentration 20 times the R.T.D.

$\frac{\text{A}}{\text{Phage } d6}$, etc., = lysogenic secondary growths resulting from treatment of Type A with latent phages.

CL = confluent lysis; ± = 6–11 plaques; *s* = plaques just visible with the naked eye; *μ* = plaques at limit of visibility with × 10 hand-lens; — = no plaque.

Another difference between the four phages in subgroup 3*a* is disclosed when Type F1 is employed as the substrate strain in type-transformation experiments (see Table 5). Phage *f*2 rapidly transforms Type F1 into F2, whereas phages *d*6, 29′ and 30′ can only effect this transformation with considerable difficulty. It is thus evident that, although very closely similar in many respects, including their serological reactions, phages *d*6, *f*2, 29′ and 30′ are nevertheless distinct phages.

Serological group 1 (see Table 8) comprises only two phages, *b*3 and 28′, which appear to be serologically identical. Up to the present it has not been possible to show that these phages, though carried by two different Vi-types of *Sal. typhi*, have a type-determining function. As these phages have identical sensitivities to physical and chemical agents and identical lytic spectra they are, for the time being, indistinguishable from each other.

Serological group 2 contains phages which have several interesting features. Phages *d*1 and *d*4 appear to be identical in all respects. They give complete cross-neutralization, are heat stable, have the same lytic spectrum and transform Type A into Type D1. Consequently, the difference between Types D1 and D4 cannot be ascribed to phages *d*1 and *d*4, but is due to some other, as yet unidentified, factor. Phage *k* has so far only been shown to make cultures resistant to the polyvalent anti-O phage, although it differs from the latter in being thermolabile. The serological relationship between phages *d*1 and *d*4, on the one hand, and phage *k* and anti-O phage no. 2 on the other, has not been found to be associated with other similarities between these phages.

A study of Table 5 will show that the identity of a Vi-type of *Sal. typhi* prepared artificially in the laboratory depends on two factors: the phage employed to carry out the change and the original Vi-phage type of the culture subjected to the treatment. When the manipulation is carried out with Type A as the substrate organism, it is not always possible to reproduce the lysogenic type from which a latent phage has been isolated. To accomplish this it is often necessary to choose one of the known specific Vi-phage types as substrate. A clue to the choice of organism of the correct type is often provided by the knowledge of the Vi-phage reactions of the original type strain and of the lytic reactions of the adapted Vi-typing phage prepared for it from Vi-phage II, bearing in mind the fact that the type-determining phages can diminish but not increase the spectrum of sensitivity of a strain to the Vi-typing phages. For example, Type 30 when first examined gave a few plaques with the routine test dilution of phage C, and Type C was found to be fully sensitive to the newly adapted Vi-typing phage 30 which was prepared by growing phage A on Type 30. When the latent phage 30′ was isolated it was found to lyse Type C and to convert it into Type 30. Knowing that phage *f*2 converted Type A into Type 29, which is sensitive to Vi-phage 30, and that the latent phage 30′ effected a similar transformation of Type A, it was predicted that if Type C were sensitive to phage *f*2, the latter would convert it into Type 30; this was found to be the case.

Since the type-determining phages can only diminish the range of phage sensitivity of a strain it would not be expected that Type E1 treated with phage *d*1 would become Type D1, as Craigie (1946) reported in his early experiments. This would necessitate not only the blocking of reactivity of the strain to Vi-phages E1 and E2, but also the opening up of the previously blocked reactivity to Vi-phages of the D group. For this reason we predicted that such treatment would result in the production of a strain untypable with the existing adapted Vi-phage preparations. This expectation proved to be correct.

Theory and practice of bacteriophage-typing

As has been pointed out previously (Felix & Anderson, 1951*a*), the fact that Vi-types can be prepared artificially does not diminish the epidemiological reliability of the Vi phage-typing method. The constancy of types characteristic of each country, and of different areas of the same country, and the fact that known carriers have been shown to excrete the same type of *Sal. typhi* over a period of years, furnish sufficient proof that the chances of the typhoid bacillus encountering type-changing phages in nature are remote.

It is necessary here to discuss the suggestion made recently by Scholtens (1950), Boyd (1950) and Boyd *et al.* (1951) of typing bacteria by identifying the phages they carry. Although the theoretical aspects of lysogenicity are, of course, of fundamental importance, a consideration of the properties of the latent phages of the typhoid bacillus described in the present paper indicates that the suggested method of typing would be unsuitable for application to *Sal. typhi*. The main reasons for this unsuitability are:

(1) Different Vi-phage types may carry the same phage, or phages so alike that their differentiation is extremely difficult. Examples are Types D6, F2, 29 and 30, all of which carry thermolabile micro-plaque phages that are serologically indistinguishable, and whose lytic spectra are closely similar, but frequently unidentifiable, because of the inconstancy of the lysis they produce on solid media. Strains of *Sal. typhi* belonging to these four Vi-phage types would almost certainly be classified as being identical by the suggested method of identifying the latent phages. In contrast, the strains are easily distinguishable from each other by the adapted Vi-typing phages.

(2) Strains from which phages have not been isolated would be grouped together under the designation of 'phage undetermined'. On this basis no less than eighteen of the thirty recognized Vi-phage types of *Sal. typhi* would have to be abandoned for the time being.

(3) The isolation and identification of the latent phages of the typhoid bacillus are often extremely difficult and time-consuming manipulations. On the other hand, the routine Vi-phage typing technique is easy and will usually give a clear-cut result in 7 hr. with *Sal. typhi*.

These points make it evident that the typing of strains of *Sal. typhi* by a procedure analogous to that suggested by Boyd (1950) and Boyd *et al.* (1951) for *Sal. typhimurium* could not be compared in simplicity, speed and reliability with the Vi-phage typing method in current international use. A recent publication by Boyd (1952) shows full recognition of this fact.

An example of the errors that may arise as the result of the use of latent phage characterization for the identification of strains of *Sal. typhi* is provided by the observations of Henderson & Ferguson (1949). These workers investigated two Vi types, D1 and D4HP56, and decided that they represented only a single type. They based this conclusion on two facts: first, that both types were being excreted by the same carrier, and secondly, that all the cultures, when examined by Craigie, yielded the same latent phage (see Henderson & Ferguson (1949)). This phage is now designated phage *d*1 and is identical with *d*4, and strain D4HP56 is identical with Type D4 described by Felix (1943). Unpublished observations of our own have shown that cultures of Type D4, maintained for a long time in the laboratory, in rare instances throw off Type D1 variants. This change is not accompanied by the loss of phage *d*1 but is apparently more closely allied to the process, or processes, of 'degradation' in the non-lysogenic specific types that lead to the emergence of Vi-phage Type A. 'Degradation' is known to occur also in chronic carriers of long standing (Felix, unpublished observations).

Type D4 is, in our experience, a valid epidemiological type that is easily distinguishable from Type D1 with the adapted Vi-typing phages, although the identification of phage *d*1 which is carried by both types will not distinguish between D1 and D4 strains. It was the identification of Type D4 and the story of a puzzling outbreak due to this type (Felix, 1943, Bradley, 1943) that helped to convince the most sceptical epidemiologists and laboratory workers of the value of the then new laboratory technique. There is no more justification for merging Types D1 and D4 on the grounds adduced by Henderson & Ferguson (1949) than there is for merging many of the other recognized types with Type A because they may, on rare occasions, throw off degraded variants reacting as Type A.

It is evident from the experiments described in this paper that the study of Vi-phage susceptibility in *Sal. typhi* and its relationship to the latent phages carried by this organism is one of considerable complexity, with the added drawback that there are many technical difficulties still to be overcome.

We gratefully acknowledge the valuable technical assistance received from Mr F. J. Flynn and Mrs E. T. Kiewe.

REFERENCES

Adams, M. H. (1950). Bacterial viruses. In *Methods in Medical Research*, **2**. Chicago: Year Book Publishers.

Anderson, E. S. (1951). The significance of Vi-phage Types F1 and F2 of *Salmonella typhi*. *J. Hyg., Camb.* **49**, 458.

Anderson, E. S. & Felix, A. (1952). Variation in Vi-phage II of *Salmonella typhi*. *Nature, Lond.* **170**, 492.

Anderson, E. S. & Felix, A. (1953). 'Degraded Vi strains' and variation in Vi-phage II of *Salmonella typhi*. *J. gen. Microbiol.* **8**, 408.

Boyd, J. S. K. (1950). The symbiotic bacteriophages of *Salmonella typhi-murium*. *J. Path. Bact.* **62**, 501.

Boyd, J. S. K. (1952). Bacteriophage-typing and epidemiological problems. *Brit. med. J.* ii, 679.

Boyd, J. S. K., Parker, M. T. & Mair, N. S. (1951). Symbiotic bacteriophage as a 'marker' in the identification of strains of *Salmonella typhi-murium*. *J. Hyg., Camb.* **49**, 442.

Bradley, W. H. (1943). An endemiological study of *Bact. typhosum* Type D4. *Brit. med. J.* i, 438.

Craigie, J. (1940). Variation of Type II Vi phage. *Proc. Third Int. Congr. Microbiol., New York*, 1939, p. 296.

Craigie, J. (1942). The present status of phage typing of *Bact. typhosum*. *Canad. publ. Hlth J.* **33**, 41.

Craigie, J. (1946). The significance and applications of bacteriophage in bacteriological and virus research. *Bact. Rev.* **10**, 73.

Craigie, J. & Felix, A. (1947). Typing of typhoid bacilli with Vi bacteriophages. *Lancet*, i, 823.

Craigie, J. & Yen, C. H. (1938). The demonstration of types of *B. typhosus* by means of preparations of Type II Vi phage. *Canad. publ. Hlth J.* **29**, 448, 484.

Felix, A. (1941). A new type of typhoid and paratyphoid vaccine. *Brit. med. J.* i, 391.

Felix, A. (1943). Experiences with typing of typhoid bacilli by means of Vi bacteriophage. *Brit. med. J.* i, 435.

Felix, A. & Anderson, E. S. (1951*a*). Bacteriophages carried by the Vi-phage types of *Salmonella typhi. Nature, Lond.* **167**, 603.

Felix, A. & Anderson, E. S. (1951*b*). Bacteriophage, virulence and agglutination tests with a strain of *Salmonella typhi* of low virulence. *J. Hyg., Camb.* **49**, 349.

Felix, A. & Callow, B. R. (1943). Typing of paratyphoid-B bacilli by means of Vi bacteriophage. *Brit. med. J.* ii, 127.

Felix, A. & Callow, B. R. (1951). Paratyphoid-B Vi-phage typing. *Lancet*, ii, 10.

Gratia, A. (1936). Des relations numériques entre bactéries lysogènes et particules de bactériophage. *Ann. Inst. Pasteur*, **57**, 652.

Hamon, Y. & Nicolle, P. (1951). Recherches sur les facteurs qui conditionnent l'appartenance des bacilles paratyphiques B aux différents types bactériophagiques de Felix et Callow. II. *Ann. Inst. Pasteur*, **80**, 496.

Henderson, N. D. & Ferguson, W. W. (1949). Investigation of bacteriophage types D1 and D4 HP 56 of *Salmonella typhi* and their latent bacteriophages. *Amer. J. Hyg.* **50**, 349.

Jude, A., Nicolle, P. & Ducrest, P. (1951). Sur la présence simultanée de deux types bactériophagiques (D1 and D6) dans une culture de *Salmonella typhi. Ann. Inst. Pasteur*, **81**, 245.

Nicolle, P., Grabar, J. & Gibert, P. (1946). Fréquence de la lysogénéité et moindre fréquence apparente de la lysosensibilité parmi les bacilles paratyphiques B. *Ann. Inst. Pasteur*, **72**, 81.

Nicolle, P., Hamon, Y. & Edlinger, E. (1951). Recherches sur les facteurs qui conditionnent l'appartenance des bacilles paratyphiques B aux différents types bactériophagiques de Felix et Callow. I. *Ann. Inst. Pasteur*, **80**, 479.

Scholtens, R. Th. (1950). Lysogenicity as a tool for bacteriophage typing of *Salmonella paratyphi B. Leeuwenhoek ned. Tijdschr.* **16**, 256.

Williams Smith, H. (1951*a*). Some observations on lysogenic strains of Salmonella. *J. gen. Microbiol.* **5**, 458.

Williams Smith, H. (1951*b*). The typing of *Salmonella thompson* by means of bacteriophage. *J. gen. Microbiol.* **5**, 472.

Williams Smith, H. (1951*c*). The typing of *Salmonella dublin* by means of bacteriophage. *J. gen. Microbiol.* **5**, 919.

EXPLANATION OF PLATE

Comparison of plaque sizes of Vi-phage A with those of latent phages 25′, 26′ and *f*2. All phages titrated on Type A and incubated for 7 hr. at 38·5° C. The heavy outer circle marks the limit of the phage-inoculated area. The light inner circle is an artefact due to the method of deposition of the bacterial lawn.

Fig. 1. Vi-phage A; × 5·5.

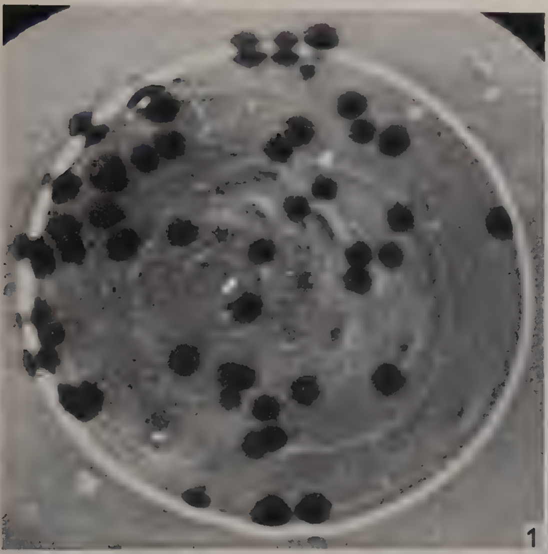
Fig. 2. Phage 25′; × 5·5.

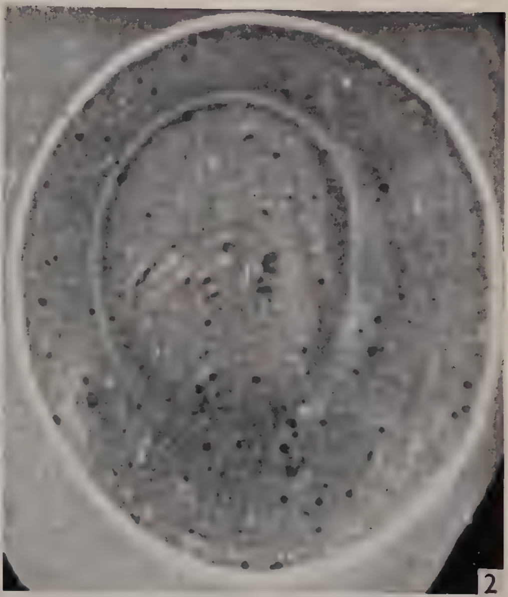
Fig. 3. Phage 26′; × 5·5.

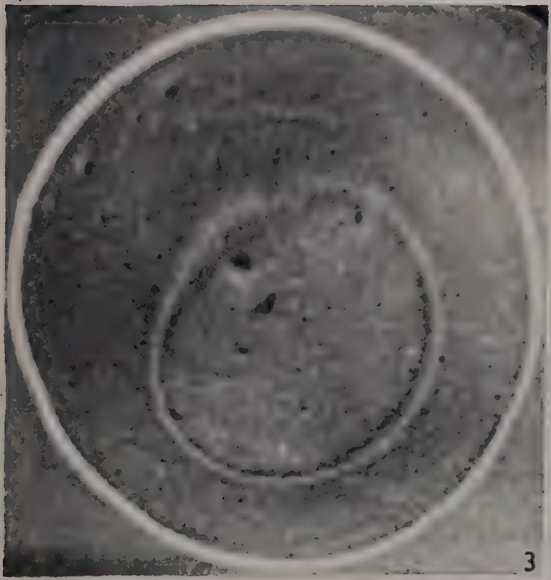
Fig. 4. Phage *f*2; × 5·5. (The area photographed contained forty plaques visible with a × 10 hand-lens; only five of the plaques are discernible in Fig. 4.)

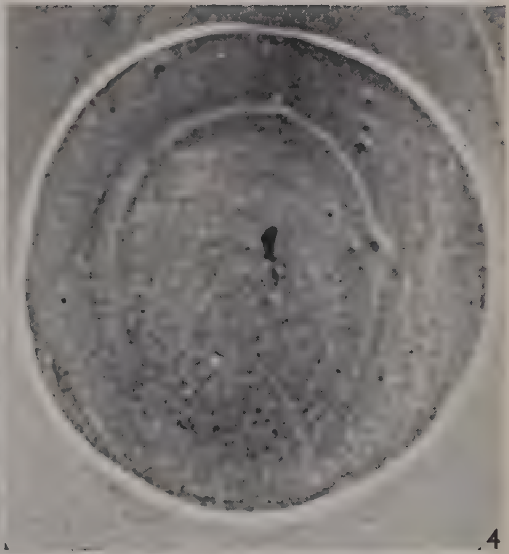
(*Received* 3 *February* 1953)

Journal of General Microbiology, Vol. 9, No. 1


1


2


3


4

E. S. ANDERSON & A. FELIX—VI TYPE-DETERMINING PHAGES OF *SALMONELLA TYPHI*. PLATE 1

Green, M. & Wilson, P. W. (1953). *J. gen. Microbiol.* 9, 89–96.

The Utilization of Nitrate Nitrogen by the Azotobacter

By MARGARET GREEN and P. W. WILSON

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin, U.S.A.

SUMMARY: Four types of utilization of nitrate nitrogen were observed with fourteen strains of the azotobacter. These were:

(*a*) Those which utilize NO_3^--N with no or only a short lag; three strains of *Azotobacter vinelandii*, a stock culture of *A. chroococcum* C-77, and seven strains of *A. chroococcum* freshly isolated from soil were in this group;

(*b*) A single strain, a stock culture of *A. chroococcum* C-44, which uses NO_3^--N only when conditions permit selection of a mutant capable of using this form of nitrogen;

(*c*) A single strain, *A. indicum* 10, which uses NO_3^--N very slowly in air and in which NO_2^--N accumulates;

(*d*) A single strain, *A. agile* 4·4, which does not use NO_3^--N. Certain other morphological and physiological differences between strains of *A. vinelandii* and the strain of *A. agile* 4·4 are pointed out which suggest that the question of the recognition of these strains as a single species be re-examined.

It has been generally accepted by workers in soil bacteriology that species of the azotobacter can grow equally well in nitrate, nitrite and ammonia and on molecular nitrogen. Robbins (1937), in a philosophical discussion dealing with the utilization of various types of nitrogen compounds by plants, stated that he knew of no reports on organisms that could use N_2 but were unable to use ammonia or nitrates. Actually, at least one report suggesting that exceptions might occur does exist. In their monograph on the azotobacter Stapp & Ruschmann (1924) noted that strains of *Azotobacter chroococcum* supplied with NH_4NO_3 used ammonia exclusively until it was exhausted, then slowly used the nitrate with accumulation of nitrite. The use of stagnant cultures in air, together with the low level of combined nitrogen (10–20 μg./ml.), suggests that the original culture did not use nitrate, but that by growth on NH_4^+ and N_2 a population was obtained from which nitrate-reducing mutants were selected.

While investigating the properties of mutants of *A. agile* selected by exposing cultures to ultraviolet light followed by penicillin screening (Lederberg, 1950), we found that the strains obtained had not only lost the ability to fix N_2 but also were unable to grow on nitrate (or nitrite), although growth was normal on ammonia or casein hydrolysate. The significance of this observation in relation to the mechanism of nitrogen fixation was definitely lessened when it was found that the parent culture likewise did not use nitrate. This unexpected finding led to an examination of stock cultures of typical strains of the azotobacter for their ability to use nitrate in the presence and absence of other sources of nitrogen. The results described in this report demonstrate that previously held concepts regarding this important aspect of the physiology of these nitrogen-fixing organisms have been incomplete and even erroneous.

METHODS

Cultures. The following stock cultures in the University of Wisconsin collection were used: *A. vinelandii* 0 (Wisconsin stock), *A. vinelandii* 3 (from Dr R. L. Starkey in 1946), *A. vinelandii* B (from Dr Dean Burk, 1940), *A. agile* 4·4 (from Dr H. A. Barker, 1947), *A. chroococcum* C-44 (from Dr R. L. Starkey, 1948), *A. chroococcum* C-77 (Wisconsin stock), and *A. indicum* 10 (from Dr R. L. Starkey, 1946). In addition, seven strains of *A. chroococcum* (C-1 to C-7) were used immediately after isolation from soil in Wisconsin.

Media and conditions of growth. Stock cultures were maintained on Burk's or Ashby's agar containing 2 % (w/v) sucrose (Wilson & Knight, 1952); strains of *A. chroococcum* grew better on Ashby's agar.

Working cultures were grown in Erlenmeyer flasks in Burk's medium containing 2 % (w/v) sucrose and were incubated at 30° on a rotary shaker (360 r.p.m.). To insure adequate aeration, the medium did not exceed one-fifth of the volume of the flask. All nitrogen sources, except nitrite which was sterilized by filtration, were autoclaved either separately or in the medium which was adjusted to pH 7·0–7·2. When it was necessary to exclude atmospheric nitrogen, the flasks were fitted with rubber stoppers and outlets to permit evacuation and replacement with hydrogen and oxygen. Adequate oxygen was supplied at intervals by evacuation and refilling.

Growth was determined turbidimetrically with an Evelyn colorimeter using a 660 mμ. filter (Wilson & Knight, 1952). Velocity constants (k values) reported are averages of at least duplicate experiments consisting of two replicates each. The cultures were grown in standard Evelyn tubes containing 11 ml. of medium and were incubated at 30° on a rotary shaker. When necessary, these tubes were fitted for hydrogen-oxygen atmospheres.

Chemical analyses. Total nitrogen was determined by the Kjeldahl method (Wilson & Knight, 1952). Nitrate nitrogen in the supernatant culture medium was reduced to ammonia by Devarda's alloy, and the ammonia was determined by titration after aeration or steam distillation into boric acid. Nitrite nitrogen was determined with the sulphanilic acid and alpha-naphthylamine reagents.

RESULTS

Velocity of growth on different sources of nitrogen. The velocity constants of growth for various species of the azotobacter when grown on N_2, NH_4^+-N and NO_3^--N are summarized in Table 1. In these experiments, 0·3 mg./ml. $(NH_4)_2HPO_4$-N and 0·2 mg./ml. KNO_3-N were used. With NO_3^--N an atmosphere of 40 % O_2 in H_2 was supplied to prevent growth on N_2; this is an unnecessary precaution with the NH_4^+-N since 0·3 mg./ml. completely inhibits fixation. A disadvantage of using the H_2+O_2 atmosphere is that O_2 soon becomes the limiting factor for growth. For example, a 500 ml. Erlenmeyer flask containing 100 ml. of medium (containing 2 g. sucrose and 30 mg.

NO_3-N) would require at least 1·5 l. of O_2 for complete oxidation of the sucrose and consequently complete utilization of the combined nitrogen. This would necessitate about twenty separate evacuations and refillings with 20 % O_2 in H_2 mixture. For this reason less medium was used with such cultures and the pO_2 raised to 0·4 atm. All inocula for these experiments were grown in N_2 for 24 hr. and were standardized so that the initial cell concentration was 10^5/ml.

Table 1. *Growth of the azotobacter in molecular nitrogen, ammonium nitrogen and nitrate nitrogen*

	N fixed (μg./ml.)		*k* value in			NO_3^--N recovery
Strain	60 hr.	Maximum*	N_2	NH_4^+-N	NO_3^--N	%
A. vinelandii 0	142	351	0·34	0·35	0·36	0
A. vinelandii 3	148	359	0·36	0·36	0·28	0
A. agile 4·4	241	338	0·38	0·40	0	101
A. chroococcum C-44	172	345	0·44	0·38	0 (0·28)	100 (0)
A. chroococcum C-77	115	237	0·40	0·37	0·44	0
A. chroococcum C-1	142	288	0·40	0·36	0·35	0
A. indicum 10	16	351	0·07	0·35	0·32	36

k, the velocity constant of growth, is equal to 2·3 times the slope of the linear portion of the curve obtained by plotting the log optical density (*OD*) against time in hours (see Figs. 1 and 2).

* Determined when 2 % sucrose was exhausted, usually 3–4 days except for *A. indicum* which required 7–10 days for maximum growth and when sucrose was often still present, and for *A. chroococcum* C-77 and C-1 strains which often did not exhaust the sucrose.

Some replicate cultures grow after a lag of 3 or more days.

Table 2. *Growth of the azotobacter on casein hydrolysate and glutamate in an atmosphere of* O_2 *in* H_2

	Source of nitrogen		
	N_2	Casein hydrolysate	Glutamate
A. vinelandii 0			
k value	0·33	0·28	0·14
Lag (hr.)	12	34	50
Optical density*	0·495	0·174	0·081
Cell N (μg./ml.)*	227	74	51
Supernate N (μg./ml.)*	8	167	191
A. agile 4·4			
k value	0·38	0·29	0·23
Lag (hr.)	7	20	40
Optical density*	0·699	0·509	0·161
Cell N (μg./ml.)*	234	191	89
Supernate N (μg./ml.)*	12	59	138

* At 48 hr. for N_2 and casein hydrolysate and 96 hr. for glutamate.

Previous studies in our own and other laboratories with *A. vinelandii* had suggested that organic sources of nitrogen such as amino-acids are inferior to the inorganic sources (Wilson, Hull & Burris, 1943). Growth on casein hydrolysate (*c.* 0·25 mg./ml.) and on glutamate (earlier work had shown this one of the better sources of the individual amino-acids) was compared with that on N_2 for a typical strain (*A. vinelandii* 0) using nitrate and one unable to use it (*A. agile* 4·4); the results are given in Table 2.

Period of lag. For interpretation of the results summarized in Table 1 the period of lag is important as well as the *k* value, which measures the velocity of growth during the logarithmic stage. Typical lags are illustrated in the curves of Figs. 1 and 2. Except for strains of *A. chroococcum,* all the cultures had shorter periods of lag and somewhat greater velocity constants in NH_4^+-N than in N_2. In contrast, *A. chroococcum* C-77 and C-1 had shorter lag periods in NO_3^--N than in N_2. Although the period of lag in NO_3^--N for all the remaining strains varied somewhat in replicate determinations, it always exceeded that of the cultures on ammonium or molecular nitrogen. The sole exception is *A. indicum* for which the results are not directly comparable with the others. First, this culture fixes N_2 extremely slowly when the initial pH is 7·0 or above, especially if small inocula are used. Secondly, below an initial pH of 7·0, although the lag period is shorter and the velocity constant of growth is higher, the heavy production of gum and the effect of pH on its production make determination of *k* values based on turbidimetric readings highly questionable.

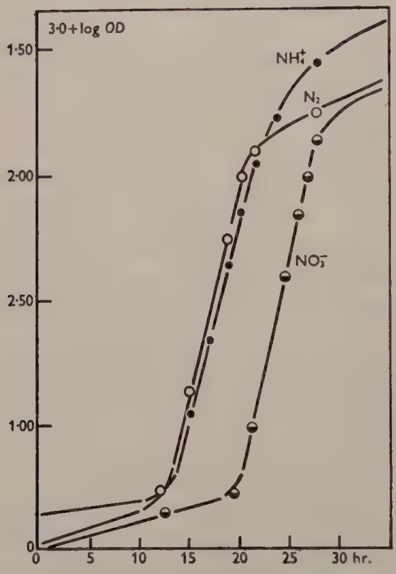


Fig. 1. Growth curves of *A. vinelandii* 0 on various sources of nitrogen. (Growth on KNO_3-N under H_2+O_2 mixture.)

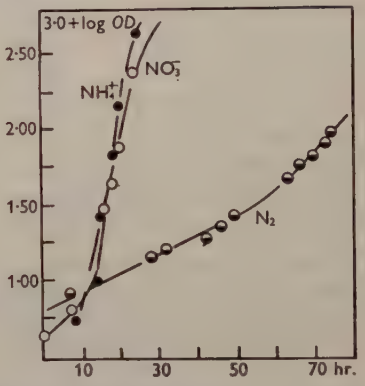


Fig. 2. Growth curves of *A. indicum* 10 in various sources of nitrogen. (Growth on KNO_3-N under H_2+O_2 mixture.)

Adaptation to nitrate. The long lag of some of the cultures in nitrate-N, together with the occasional use of nitrate-N by *A. chroococcum* C-44, could be accounted for either by the formation of adaptive enzymes or the selection of a mutant utilizing nitrate. The occurrence of both might be favoured by growing the cultures in the presence of nitrate, together with other forms of nitrogen. The initial trials were made with strains growing in 0·166 mg./ml. NH_4NO_3-N in air. All cultures grew well under these conditions except

A. chroococcum C-44 and *A. agile* 4·4. The pH in these two cultures fell to about 5·0 because of the utilization of the basic ammonium ion. Under these conditions N_2 cannot be fixed, and growth was stopped without detectable use of the nitrate. The results with *A. indicum* deserve special mention. After 6 days no nitrate had been used, and the pH had dropped to 3·2. This unusual species of the azotobacter can survive even this low pH (Starkey, 1939), so that in 8 weeks it had metabolized about two-thirds of the nitrate, and the pH had correspondingly returned toward neutrality. These three cultures were accordingly retested, using only 0·7 mg./ml. NH_4NO_3-N with the results summarized in Table 3. Under these conditions, only *A. agile* 4·4 did not use NO_3^--N in 4 days.

Table 3. *Utilization of nitrate*-N *from small concentrations of* NH_4NO_3 *in air*

Strain	Age (days)	Final pH	Optical density	Initial NO_3^--N (μg./ml.)	NO_3^--N recovery (%)
A. agile 4·4	4	6·5	0·921	35	104
A. chroococcum C-44	1	6·1	0·215	35	104
	2	6·3	0·367	35	64
	4	6·4	1·046	35	0
A. indicum 10	1	6·2	0·553	35	25
	4	4·0	1·398	35	0

Another successful method for obtaining strains of *A. chroococcum* C-44 capable of using nitrate-N was by serial transfers using 1/10 vol. of the previous culture as inoculum in air in a medium containing KNO_3-N (see Table 4). *A. agile* 4·4 did not use NO_3^--N; the behaviour of *A. indicum* 10 under these conditions will be discussed in the next section on the formation of nitrite.

To determine if the utilization of nitrate-N resulted from a prolonged enzymic adaptation to nitrate similar to that taking place within a few hours

Table 4. *Serial transfers of strains of azotobacter on* KNO_3-N *in air*

Strain	Transfer no.	Age (days)	Final pH	Optical density	NO_2^--N (μg./ml.)	Initial NO_3^--N (μg./ml.)	NO_3^--N recovery (%)
A. chroococcum C-44	2	2	7·5	1·301*	0	319	102
	5	7	7·7	1·523*	0	229	104
	6	7	7·7	1·699*	0	229	86
	7	3	8·2	2·000*	0	229	1
A. agile 4·4	7	7	7·6	1·000	0	166	100
A. indicum 10	3	5	6·8	0·180	50	229	78†
	7	3	7·0	0·215	52	225	75†

* Black pigment present.

† Corrected for NO_2^--N present; note that the recovery of NO_3^--N plus NO_2^--N is about 100 per cent and therefore N_2 was serving as the source of nitrogen.

When *A. chroococcum* C-44 (transfer no. 6 above) was inoculated into 0·3 mg./ml. KNO_3-N in a H_2+O_2 atmosphere, all the nitrate-N disappeared in 4 days. *A. indicum* (transfer no. 7) used only 27 % of the nitrate-N in 17 days under H_2+O_2 and 0·053 mg./ml. NO_2^--N were present. *A. agile* (transfer no. 7) failed to grow in nitrate-N when N_2 was excluded. Also continuous cultivation of *A. agile* through seventy-eight transfers in 0·15 mg./ml. NH_4NO_3-N in air did not result in utilization of nitrate-N.

with *A. vinelandii* 0 (Burris & Wilson, 1946), Burk's medium containing 0·166 mg./ml. KNO_3-N was inoculated with all the strains of the azotobacter and incubated under 40 % O_2 in H_2. If small inocula were used (10^3 cells/ml.), *A. agile* 4·4 and *A. chroococcum* C-44 (occasional exceptions) failed to grow. *A. chroococcum* C-44, but not *A. agile* 4·4, grew when the size of the inoculum was increased to 10^7 cells/ml. All other strains, except *A. indicum*, used up to 0·02 mg./ml. KNO_3-N within 4 days with either large or small inocula when kept in the H_2+O_2 atmosphere. *A. indicum* used NO_3^--N but at a much slower rate.

Formation of nitrite. Although *A. indicum* is capable of using nitrate, under certain conditions, the utilization is incomplete in a medium containing approximately 0·15 mg./ml. total-N. Decreasing the initial pH of the medium did not alter this result in contrast to its effect on the fixation of N_2. This incomplete utilization evidently arises from accumulation of toxic quantities of nitrite in the medium. For example, with 0·15 mg./ml. NO_3^--N initially present, as much as 0·05–0·06 mg./ml. NO_2^--N accumulated (its toxicity may be accentuated by the low pH frequently observed in cultures of *A. indicum* 10). Variation of the pO_2 (0·0–0·5 atm.) or replacement of the hydrogen + oxygen atmosphere with one of helium + oxygen did not influence the accumulation. Serial transfers in nitrate-N did not select a culture which could use nitrite-N more readily.

Only occasionally with the other strains did nitrite accumulate: it did so with *A. vinelandii* 0 during the first few hours of growth on 0·35 mg./ml. NH_4NO_3-N in air, and with this culture and *A. chroococcum* C-44 when oxygen was limiting (H_2+O_2 atmosphere).

DISCUSSION

All the stock strains of *A. vinelandii* tested grew readily on nitrate-N, but neither the parent nor a derived strain (the mutant unable to use N_2) of *A. agile* 4·4 would use this source of nitrogen. Unsuccessful efforts to secure utilization of nitrate by this culture in addition to those summarized in the results included: (*a*) a tenfold increase in content of molybdenum in the medium—this element is believed to be important in nitrate reduction (Steinberg, 1939)—and (*b*) reduction of the pO_2. The classical method of genetics, the use of mutagenic agents, was not attempted. The inability to use nitrate-N by this culture does not arise from a lack of nitrate reductase alone since the culture likewise cannot use nitrite-N even when present in a non-toxic concentration of 0·02 mg. N/ml.

One culture (C-44) of the nine tested strains of *A. chroococcum* did not use nitrate-N, but strains able to use nitrate were readily obtained from this culture, probably by selection of mutants. Another culture, *A. indicum* 10, used nitrate-N slowly in air and nitrite-N accumulated. Since all of the cultures grew well on 0·35 mg. NO_3-N/ml. in air, regardless of whether this source of nitrogen or N_2 was used, it is easily understood why inability to use nitrate-N by certain strains of the azotobacter was so long overlooked.

Finally, as previously reported, species of azotobacter grow more readily on N_2 and NH_4^+-N than on organic nitrogen as evidenced by shorter periods of lag and greater k values. Nevertheless, the growth on a complex mixture of amino-acids such as casein hydrolysate is considerably better than we had previously reported (Wilson *et al.* 1943); this is particularly true of *A. agile* 4·4.

Although probably none of these results could be generalized when so few strains have been used, the observed difference in the behaviour of the single strain of *A. agile* 4·4 and the three strains of *A. vinelandii* emphasizes the discrepancies that have resulted in a disparity of opinion regarding the validity of classifying *A. vinelandii* as a strain of *A. agile* (Hofer, 1948). Some differences which we and others (Winogradsky, 1938; Kluyver & van Reenan, 1933) have observed between *A. vinelandii* and *A. agile* are:

(1) Morphologically, *A. agile* 4·4 is a much larger organism than any of our strains of *A. vinelandii*. *A. agile* 4·4 is particularly characterized by prominent intracellular particles (Eisenstark, Ward & Kyle, 1950), some of which stain with dyes considered to be specific for mitochrondria. Capsular material and gum are much more evident in *A. agile*, and the cell is much more resistant to breakage and does not autolyse readily in old cultures.

(2) Physiologically, *A. agile* 4·4 has a much shorter lag than has *A. vinelandii* when grown on N_2 or NH_4^+-N, although the total quantity of N_2 fixed and the efficiency of fixation do not differ markedly among the cultures. In addition to being unable to use nitrate-N, this strain of *A. agile* does not oxidize mannitol; *A. vinelandii* will use this source of carbon although the enzyme system is apparently adaptive.

Whether these differences are sufficient for recognition of two species could be debated and certainly their significance is dependent upon a wider survey of representative types.

It may also be mentioned that two characteristics of the azotobacter given by Hofer (1948) are not met by any of the cultures in our collection. The statement that members of the family grow best on media deficient in nitrogen has not been borne out by any modern physiological studies in which ammonia and urea have been used. The apparently contradictory statement that *A. chroococcum* as a species is stimulated by non-toxic concentrations of nitrate probably is based on early experiments in which the physiology of nitrogen fixation, specifically the role of molybdenum, was not understood. Finally, the frequently used characteristic of pigmentation for differentiation of species is open to question since the presence of many of the pigments depends in a large part on the physiological environment, notably levels of iron, molybdenum and aeration (Wilson & Knight, 1952). Because of these difficulties and the almost total lack of information based on modern physiological techniques, except for a very few strains, we prefer for historical reasons (Winogradsky, 1938; Kluyver & van Reenan, 1933), as well as those discussed in this section, to maintain the two species, *A. agile* and *A. vinelandii,* until more compelling arguments are given for their merging. At present it is evident that this field once more needs a thoroughgoing survey analogous to that made 30 years ago by Stapp & Ruschmann (1924).

REFERENCES

Burris, R. H. & Wilson, P. W. (1946). Ammonia as an intermediate in nitrogen fixation by *Azotobacter*. *J. Bact.* **52**, 505.

Eisenstark, A., Ward, Jn. C. B., & Kyle, T. S. (1950). A study of large bodies in *Azotobacter agile*. *J. Bact.* **60**, 525.

Hofer, A. W. in *Bergey's Manual of Determinative Bacteriology* (1948). 6th ed. Edited by Breed, R. S., Murray, E. G. D. & Hitchens, A. P. Baltimore, Maryland: Williams and Wilkins Co. p. 220.

Kluyver, A. H. & Reenen, W. J. van (1933). Über *Azotobacter agilis* Beijerinck. *Arch. Mikrobiol.* **4**, 280.

Lederberg, J. (1950). Isolation and characterization of biochemical mutants of bacteria. *Meth. med. Res.* **3**, 5.

Robbins, W. J. (1937). The assimilation by plants of various forms of nitrogen. *Amer. J. Bot.* **24**, 243.

Stapp, C. & Ruschmann, G. (1924). Zur Biologie von *Azotobacter*. *Arb. biol. Abt.* (*Anst.—Reichsanst.*), *Berl.* **13**, 305.

Starkey, R. L. (1939). The influence of reaction upon the development of an acid-tolerant *Azotobacter*. *Trans. Third Comm. Soc. int. Soil Sci.* A, p. 142.

Steinberg, R. A. (1939). Effects of nitrogen compounds and trace elements on growth of *Aspergillus niger*. *J. agric. Res.* **59**, 731.

Wilson, P. W., Hull, J. F. & Burris, R. H. (1943). Competition between free and combined nitrogen in nutrition of *Azotobacter*. *Proc. Nat. Acad. Sci., Wash.* **29**, 289.

Wilson, P. W., & Knight, S. G. (1952). *Experiments in Bacterial Physiology*. Minneapolis, Minnesota: Burgess Publishing Co.

Winogradsky, S. (1938). Études sur la microbiologie du sol et des eaux. Sur la morphologie et l'œcologie des *Azotobacter*. *Ann. Inst. Pasteur*, **60**, 351.

(*Received* 7 *February* 1953)

Levy, E., Rippon, J. E. & Williams, R. E. O. (1953). *J. gen. Microbiol.* 9, 97–100.

Relation of Bacteriophage Pattern to some Biological Properties of Staphylococci

By E. LEVY

Bacteriology Department, St George's Hospital Medical School, London

JOAN E. RIPPON and R. E. O. WILLIAMS

Staphylococcal Reference Laboratory, Public Health Laboratory Service, Colindale, London, N.W. 9

SUMMARY: Representative coagulase-positive staphylococci that had been examined for their production of diffusible antigens, α- and β-haemolysins, fibrinolysin and pigment, were typed by bacteriophage. Among strains from human sources there was no significant association between the phage group and the other properties. Strains of staphylococci obtained from animals seemed, however, to form a different biological group from those from human beings.

Although it is known that certain phage-types of staphylococci are particularly common as the cause of some staphylococcal diseases, such as food poisoning (Williams, Rippon & Dowsett, 1953), there is no similar information on the association of these types with any other physiological property except penicillinase production, although Smith (1948) reported differences between human and animal strains. We therefore determined the phage types of a series of staphylococci whose biological properties had previously been studied (Elek & Levy, 1950*a*, *b*).

METHODS

The phage typing was carried out by the methods already described (Williams & Rippon, 1952) and the strains were classified on the basis of the pattern of lysis by the phages into three groups (Williams & Rippon, 1952, Williams, Rippon & Dowsett, 1953) known as I (the '52 group'), II (the '3A group') and III (the '6/47 group'). Strains that were lysed by some of the phages, but which could not be fitted into this classification were called 'unclassifiable'. Some of the strains proved resistant to all phages used at their routine test dilution, but sensitive to undiluted phages; these are called 'partially typable'. Others were resistant even to the undiluted phages and called 'completely untypable'.

The techniques employed for the determination of diffusible antigens, α- and β-haemolysin production and pigment formation, together with details of the origins of the strains have been fully described in previous publications (Elek & Levy, 1950*a*, *b*).

For the determination of fibrinolysin, double strength basal medium as described in these papers was employed and mixed with equal quantities of human plasma. The poured plates were heated at 56° until turbid, to precipitate fibrin. After inoculation they were incubated in an atmosphere of 30 % carbon dioxide and 70 % air at 37° for 24 hr. All strains were first tested by spot inoculation on to these plates. A circular area of clearing wider than the diameter of the growth was regarded as indicating fibrinolysin production. Strains negative by this test were recorded as fibrinolysin-negative. The positive reactions were confirmed on similar plates incorporating strips of filter-paper soaked in Burroughs Wellcome Refined Staphylococcal Globulins (1250 units α-antitoxin/ml.). Streak inoculations were made at right angles to the strips, up to six organisms being tested on one plate. The plates were then treated as described for the spot inoculation procedure. This routine was adopted to see whether any differentiation of fibrinolysins analogous to that of the haemolysins would be possible. This did not prove to be the case, and with all the strains found fibrinolysin-positive by the spot method, the zones of clearing in the streak method were inhibited by the antiserum.

RESULTS

Of the 254 strains tested, fifty were derived from animal sources (Table 1); with one exception the animal strains were in phage group III, or were unclassifiable or untypable. No fewer than sixteen of the twenty-five strains that were not lysed even by the undiluted phages were from animals.

Table 1. *Phage group of strains from human and animal sources*

		Percentage of strains	
Phage group	No. of strains tested	Human sources	Animal sources
I	85	98·8	1·2
II	26	100·0	0
III	68	75·0	25·0
Unclassifiable	12	66·7	33·3
Partially typable	38	68·4	31·6
Completely untypable	25	36·0	64·0
Total	254	80·3	19·7

Table 2. *Pigment production by staphylococci of different phage groups*

		Percentage with colonies				
				Golden		
Phage group	No. of strains	White	Off white	1	2	3*
I	85	3·5	0	21·2	42·4	32·9
II	26	3·8	15·4	19·2	19·2	42·4
III	68	14·7	11·7	25·0	32·4	16·2
Unclassifiable	12	0	16·7	33·3	25·0	25·0
Partially typable	38	0	10·5	26·3	42·2	21·0
Completely untypable	25	12·0	12·0	40·0	28·0	8·0
Total	254	6·7	8·3	25·2	35·0	24·8

* Golden pigment graded 1, 2, 3 to denote increasing depth of colour.

Strains of groups I and II, which were almost exclusively from human sources, did not differ in any of the characteristics studied; and none of the groups differed in their mean number of flocculation lines (Tables 2 and 3).

Group III and the completely untypable group contained a greater proportion than the others of strains that were poorly pigmented, or produced β-haemolysin, and they also contained a smaller proportion than the others of fibrinolysin-producing strains. The two last characteristics were also noted in the unclassifiable and the partially typable groups. It seems that these differences could be explained by the fact that they are characteristic of animal strains, which were common in these groups (see Table 4).

Table 3. *Production of flocculation lines, haemolysin, and fibrinolysin by staphylococci of different phage groups*

	Flocculation lines		Haemolysins			Fibrinolysin	
				% producing			
Phage group	No. of strains tested	Mean no. lines	No. of strains tested	α	β	No. of strains tested	% positive
I	85	6·3	85	94·2	7·1	82	81·7
II	26	6·5	26	96·2	7·7	25	88·0
III	68	5·9	68	91·2	32·4	65	75·5
Unclassifiable	12	5·8	12	91·7	50·0	12	50·0
Partially typable	38	6·1	38	92·1	36·8	36	66·7
Completely untypable	25	5·3	25	88·0	64·0	24	62·5
Total	254	6·02	254	92·5	26·0	244	75·0

Table 4. *Comparison of staphylococci from human and animal sources*

	β-haemolysin		Fibrinolysin		Pigmentation		
Source	No. tested	% positive	No. tested	% positive	No. tested	% white and off white	% golden
Human	204	10·3	204	81·3	204	8·8	91·2
Animal	50	90·0	49	34·7	50	40·0	60·0

The fact that the animal strains are less often typable than the human is not surprising, since the phages were collected principally for the identification of human strains. Smith (1948) also showed that strains from cows frequently had the phage pattern 42D, which is relatively rare among human strains, and that strains from sheep were quite untypable with the set of phages used for typing human strains, although susceptible to other phages. These facts and the other known differences in the metabolic activities of human and animal strains, some of which we have confirmed, suggest that the strains from animals form a different biological group from those derived from human sources.

The substance of this paper formed part of a thesis submitted by E. Levy for the Ph.D. degree in the University of London, July 1952.

REFERENCES

Elek, S. D. & Levy, E. (1950*a*). Diffusible antigens in staphylococcal cultures. *Brit. J. exp. Path.* **31**, 358.

Elek, S. D. & Levy, E. (1950*b*). Distribution of haemolysins in pathogenic and non-pathogenic staphylococci. *J. Path. Bact.* **62**, 541.

Smith, H. W. (1948). The typing of staphylococci of animal origin by the bacteriophage method. *J. comp. Path.* **58**, 179.

Williams, R. E. O. & Rippon, J. E. (1952). Bacteriophage typing of *Staphylococcus aureus. J. Hyg., Camb.* **50**, 320.

Williams, R. E. O., Rippon, J. E. & Dowsett, L. M. (1953). Bacteriophage typing of strains of *Staphylococcus aureus* from various sources. *Lancet,* **i**, 510.

(*Received* 12 *February* 1953)

COWAN, S. T. (1953). *J. gen. Microbiol.* 9, 101–109.

Micromethod for the Methyl Red Test

BY S. T. COWAN
National Collection of Type Cultures, Colindale Avenue,
London, N.W. 9

SUMMARY: A micromethod for the methyl red (MR) test was worked out for suspensions of bacterial cells. The most important factors in the test are (1) the density of the cell suspension; (2) the ratio of glucose to buffer concentrations. Adaptation of the cells to glucose is essential for the success of the test, and cells from glucose-free media are not satisfactory. At the optimal glucose : buffer ratio *Bacterium coli* and *Klebsiella aerogenes* suspensions have different effects on the final pH value attained in the system.

Because the MR microtest is dependent on a fine balance between glucose and buffer concentrations, and is so greatly affected by even twofold differences in cell concentration, it is not really suitable for routine work in the characterization of bacteria.

The methyl red (MR) test was developed by Clark & Lubs (1915) who rather optimistically said that 'Simple conditions have been found under which metabolism can be so controlled that the hydrogen ion concentrations of cultures of one group can be made to diverge widely from those of the other group' (p. 173). The test measures the final acidity of a culture in a buffered medium containing glucose and peptone, but the pH value recorded at the time of the test is not necessarily the lowest value attained during growth. In the early stages of glucose fermentation sufficient acid is produced by both *Bacterium coli* and *Klebsiella aerogenes* (syn. *Bact. aerogenes*, *Aerobacter aerogenes*) to turn methyl red indicator orange or red (MR-positive), but on further incubation *K. aerogenes* breaks down the pyruvic and other acids, and produces a reversion in the reaction of the culture towards neutrality; methyl red indicator becomes yellow (MR-negative). The timing of this sequence,

starting pH (*c.* 7) → low pH (*c.* 5) → higher pH (6 or more),

will be affected by the medium, particularly the buffering power of the peptone and phosphate, by the temperature and duration of incubation, and by the strain. It is surprising, therefore, that a greater attempt has not been made to standardize the test.

At first sight it did not appear that the MR test could be fitted into the range of microtests on suspensions developed by Clarke & Cowan (1952). In cultures growing in a complex medium such as glucose phosphate peptone broth, more than one enzyme system will be in action and each may have some effect on the pH value of the culture; in particular NH_3 production from peptone can be expected to prevent the development of an extremely low pH value. On the other hand, in living but non-multiplying (static) suspensions, glucose fermentation has a simpler effect on the pH value; the introduction of phosphate buffer slows the initial fall and may prevent the ultimate inactivation of the enzyme system. Contrary to expectation, a workable MR microtest was developed from the modified fermentation method (Cowan, 1953).

MATERIALS AND METHODS

Strains. Classical strains of *Bact. coli*, including Escherich's original strain (NCTC 86), and of *K. aerogenes*, including two reputed descendants of Pfeiffer's *Bacillus capsulatus* (NCTC 243 and 418), were supplemented by other well-authenticated strains from Dr E. Windle Taylor of the Metropolitan Water Board's laboratory, various serotypes of the Escherichia and Klebsiella groups from Dr F. Kauffmann, strains from Dr T. Richards of Reading University, and a series of coliform bacteria collected for another purpose.

Media. The basal medium (LA) had the following composition: Lemco 1 %, NaCl 0·5 %, Evans peptone 1 %, New Zealand agar 1 %; pH 7·6. Glucose agar (GA) was LA + 1 % glucose.

Cultures. Except where stated otherwise, cultures were grown for 18–22 hr. at 30° on GA.

Suspensions. Cultures were washed off solid medium into sterile distilled water, centrifuged and the deposit resuspended in distilled water.

Buffer. Sørensen's phosphate buffer, pH 6·8, was used at various molar concentrations.

Indicators. Methyl red (0·04 %) and bromcresol purple (0·04 %) were made up in 98 % ethanol.

Methyl red (MR) *test.* Tests were made in 65 × 10 mm. tubes, to which were added 0·5 ml. phosphate buffer pH 6·8, 0·05 ml. glucose, and 0·05 ml. suspension. Concentrations of buffer, glucose and cell suspension varied in different experiments. Except where stated, the mixtures were kept at 37° (water-bath), and at intervals tubes were removed for test; to these 0·06–0·08 ml. methyl red solution was added, the tubes shaken and the colours compared with a standard. In many experiments duplicate sets were made, and to each tube of one set 0·025 ml. bromcresol purple was added, and pH values were read at intervals. Capillator sets of standards (British Drug Houses, Ltd., London) for methyl red and bromcresol purple were used for experiments in which the result was expressed as a pH value.

RESULTS

Change in pH value during glucose fermentation

In the fermentation of glucose by *Bact. coli* suspensions there was a progressive increase in acidity and fall in pH value; on the other hand, with *K. aerogenes* there was a quicker initial fall in pH, and later a gradual reversal of reaction, presumably because of the breakdown of pyruvic acid. Table 1 shows the results of an experiment in which 0·1 M-glucose was fermented in the presence of 0·025 M-phosphate buffer, pH 6·8. Within the first hour *K. aerogenes* had lowered the pH much more than had *Bact. coli*, but after the second hour the reversal of reaction was apparent. From this experiment it is seen that the changes in pH value closely parallel those of cultures growing in glucose phosphate peptone broth. Translated into terms of the MR test a reaction of pH 5·0 or less is MR +, 5·8 or above is MR −, and values between 5·0 and 5·8 are MR ± or doubtful. With a doubtful range extending so widely it may be advisable to use bromcresol purple to supplement the information given by methyl red.

Table 1. *Changes in pH value during the breakdown of glucose by* Bacterium coli *and* Klebsiella aerogenes *suspensions*

0·025 M-buffer, pH 6·8, 0·5 ml.; 0·1 M-glucose, 0·05 ml.; suspension, 0·05 ml.

Time (hr.)	*Bact. coli* (NCTC 86)	*K. aerogenes* (NCTC 243)
	pH values	
0	6·8	6·8
1	6·0	5·4
2	5·4	5·4
3	5·4	5·8
4	5·2	5·8
6	5·4	6·0
24	4·8	6·0

Importance of buffer in test

Bact. coli (NCTC 8169) and *K. aerogenes* (NCTC 8348) were grown on LA and GA and suspensions were made from each culture. Suspensions were added to 0·1 M-glucose and diluting fluid which, in three cases was phosphate buffer pH 6·8, and in the fourth was distilled water. Hourly tests up to 6 hr. and at 24 hr. made by the addition of methyl red showed (Table 2) that both strains were always MR-positive (MR +) when water was the diluent and

Table 2. *Effect of growth medium, buffer concentration and duration of incubation on methyl red microtest*

Buffer pH 6·8, or water, 0·5 ml.; 0·1 M-glucose, 0·05 ml.; suspension, 0·04 ml.
Incubation at 37°

		Suspension from growth on													
		LA							GA						
	Buffer concentration	Time of reading (hr.)													
Strain	(M)	1	2	3	4	5	6	24	1	2	3	4	5	6	24
NCTC 8169,	0·07	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Bact. coli	0·025	−	±	+	+	+	+	+	±	+	+	+	+	+	+
	0·01	±	+	+	+	+	+	+	+	+	+	+	+	+	+
	Water	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NCTC 8348,	0·07	−	−	−	−	−	−	−	−	−	−	−	−	−	−
K. aerogenes	0·025	−	±	+	+	+	+	+	±	±	−	−	−	−	−
	0·01	±	+	+	+	+	+	+	+	+	+	+	±	±	−
	Water	+	+	+	+	+	+	+	+	+	+	+	+	+	+

MR test: + = red, ± = orange, − = yellow.

MR-negative (MR –) with strong buffer (0·07 M); that the distinction between the strains was only seen in suspensions made from growth on GA; and that it was best seen in buffer at 0·025 M concentration, and after 24 hr. incubation.

Table 3. *Effect of suspension concentration and volume of* 0·1 M-*glucose in the test*

0·025 M-phosphate buffer, pH 6·8, 0·5 ml.; 0·1 M-glucose, 0·04–0·06 ml.; suspension 0·05 ml.

Strain	Suspension $\times 10^9$/ml.	0·1 M-glucose 0·04 ml.	0·05 ml.	0·06 ml.
NCTC 86, *Bact. coli*	16	–	+	+
	8	–	+	+
	4	–	+	+
	2	–	–	–
NCTC 243, *K. aerogenes*	16	–	–	–
	8	–	–	–
	4	–	–	–
	2	–	–	±

+ = pH 5·0 or less; ± = pH *c.* 5·6; – = 5·8 or more. Readings after 24 hr. at 37°.

Other preliminary experiments showed that, at one buffer concentration, the result was influenced by suspension concentration and by the volume of 0·1 M-glucose added (Table 3). It became clear that in the application of the test to routine work, roughly calibrated pipettes, adequate for the other microtests, would not be reliable and that correctly graded pipettes were essential.

Relation between glucose and buffer concentrations

Phosphate buffer, pH 6·8, was kept at a constant volume of 0·5 ml., glucose at 0·05 ml., and suspension at 0·05 ml., and the concentrations were varied. The general plan was to set up the experiment in duplicate; bromcresol purple was added as an internal indicator to one set and readings were taken at intervals by comparing the colour with the capillator colour card for that indicator. After 24 hr. methyl red was added to the second set and the colours matched against the appropriate capillator card. In the range in which the indicators overlapped, the readings were averaged.

In most experiments the suspension was kept constant and both the buffer and glucose concentrations were varied, in a few experiments all three were varied (Table 4). When the suspension is constant and is at least equivalent in density to 8×10^9 *Bact. coli*/ml., there is a ratio of glucose : buffer concentrations at which the distinction between *Bact. coli* and *K. aerogenes* can be seen. When glucose is proportionately greater both species produce more acid and are indistinguishable; proportionately more buffer again masks the differences. The optimal glucose : buffer ratio generally holds good between 0·1 and 0·8 M-glucose and 0·025 and 0·2 M-phosphate buffer; ignoring the difference in volumes and expressing the ratio in terms of molar concentrations, it is 4 : 1; when allowance is made for the difference in volume the optimal proportion is 4 : 10. Below 0·1 M-glucose this ratio does not hold, and even at 0·1 M-glucose an occasional strain will show an anomalous result.

When the suspension density is less than 8×10^9 *Bact. coli*/ml. the distinction between *Bact. coli* and *K. aerogenes* is lost, even at the optimal glucose : buffer ratio.

Table 4. *Experiment to show relation between concentrations of glucose and buffer, and the effect of suspension concentration*

Buffer pH 6·8, 0·5 ml.; glucose solution, 0·05 ml.; suspension, 0·05 ml.
Test read after 24 hr. at 37°

Strain	Glucose concentration (M)	Suspension concentration (cells/ml.)							
		16×10^9				8×10^9			
		Concentration of buffer (M)							
		0·1	0·05	0·025	0·0125	0·1	0·05	0·025	0·0125
NCTC 86, *Bact. coli*	0·4	5·0	4·6	4·6	4·6	4·8	4·6	4·6	4·6
	0·2	6·2	5·0	4·6	4·6	6·2	5·0	4·6	4·6
	0·1	6·6	6·4	5·2	4·6	6·6	6·4	5·4	4·6
	0·05	6·6	6·6	6·4	5·8	6·6	6·4	6·4	5·8
NCTC 243, *K. aerogenes*	0·4	6·2	5·5	4·6	4·6	6·2	5·5	4·6	4·6
	0·2	6·4	6·0	5·4	4·8	6·4	6·2	5·5	4·6
	0·1	6·6	6·4	5·9	5·4	6·6	6·4	6·0	5·5
	0·05	6·6	6·6	6·4	5·9	6·6	6·6	6·4	6·0

Strain	Glucose concentration (M)	Suspension concentration (cells/ml.)							
		4×10^9				2×10^9			
		Concentration of buffer (M)							
		0·1	0·05	0·025	0·0125	0·1	0·05	0·025	0·0125
NCTC 86, *Bact. coli*	0·4	5·8	4·8	4·6	4·6	6·4	6·0	5·1	4·6
	0·2	6·2	5·1	4·6	4·6	6·6	6·0	5·1	4·6
	0·1	6·4	6·2	5·6	4·6	6·6	6·1	5·1	4·6
	0·05	6·6	6·6	6·4	6·0	6·6	6·6	6·4	5·8
NCTC 243, *K. aerogenes*	0·4	6·2	5·5	4·6	4·6	6·4	6·2	5·0	4·6
	0·2	6·4	6·0	5·4	4·8	6·4	6·2	5·0	4·6
	0·1	6·6	6·4	6·2	5·5	6·6	6·4	5·8	5·3
	0·05	6·6	6·6	6·4	6·2	6·8	6·8	6·6	6·0

pH values estimated with methyl red and bromcresol purple; a mean was taken in the range of overlap.

Squared figures have the same glucose : buffer ratio.

Effect of temperature

Cultures were grown at 30 and 37°, and suspensions made from each were tested in water-baths at 30 and 37°. The temperature of the test had little effect on the final pH value attained in 24 hr. at the optimal glucose : buffer ratio, but the temperature at which the culture had been grown occasionally had some influence.

Some strains, such as NCTC 8835, when grown at 30° yielded suspensions which produced a pH of about 6·0 (MR −) and when grown at 37° the suspensions produced a pH of 5·2–5·8, that is in the MR doubtful range. Other strains grown at the two temperatures yielded suspensions that hardly differed from each other; any effect was a slightly lower pH from cultures grown at 37°. What differences there are seem to relate to the optimal temperature for growth which, for most coliforms, is nearer 30° than 37°.

Effect of growth medium

Adaptation of the cells to glucose seems to be a prerequisite for the MR microtest; without such adaptation, as with suspensions from growth on LA. *K. aerogenes* remained MR + (Table 2). The effect of different carbohydrates was compared by growing *Bact. coli* and *K. aerogenes* on LA + glucose, lactose, maltose or mannitol, and preparing suspensions from the resulting growths. In the microtest made at different glucose and buffer concentrations, *K. aerogenes* suspensions from LA + glucose produced a pH of 6·2 (i.e. MR −) in all tubes at the optimal glucose : buffer ratio. On the other hand, the final pH value attained by suspensions from the other sugar media was not uniform, even at the optimal ratio, and it appeared that adaptation to glucose was incomplete (Table 5).

Most experiments were made on suspensions grown on LA and GA, but similar results were obtained when the organisms were grown on 'richer' media such as Hartley digest agar and the same medium + glucose. Hoogerheide (1940) showed that klebsiellas were less mucoid on nitrate media, and because of difficulties in spinning out the cells trials were made of suspensions from LA + 1 % KNO_3 and LA + 1 % KNO_3 + 1 % glucose. Table 6 shows that LA + KNO_3 gave suspensions that behaved as those from LA, i.e. a *K. aerogenes* suspension maintained a low pH (MR +) and did not produce a reversal of reaction; LA + KNO_3 + glucose gave suspensions that behaved like GA, and the pH reverted to the alkaline side of methyl red (MR −).

DISCUSSION

It is hardly appropriate to call a test the 'MR' when bromcresol purple may be used as an alternative indicator, but the application of the MR test to cultures of coliform bacteria is so well established that to change the name of the test would only cause confusion. The MR test merely indicates the pH value of a culture grown in a glucose phosphate peptone medium for an unspecified time at an unspecified temperature, and individual workers have set their own

Table 5. *The effect of adaptation to different sugars on* pH *value reached in the fermentation of glucose by* Klebsiella aerogenes (*NCTC* 8348) *and by* Bacterium coli (*NCTC* 8169)

		K. aerogenes			*Bact. coli*		
		Buffer concentration (pH 6·8)					
Suspension from growth on	Glucose concentration (M)	0·1 M	0·05 M	0·025 M	0·1 M	0·05 M	0·025 M
		pH value after 24 hr. at 37° (bromcresol purple)					
Lemco agar (LA)	0·4	5·2	5·2	5·2	5·2	5·2	5·2
	0·2	6·0	5·2	5·2	6·0	5·2	5·2
	0·1	6·4	6·2	5·2	6·4	6·2	5·2
LA + glucose	0·4	6·2	5·8	5·2	5·2	5·2	5·2
	0·2	6·4	6·2	5·8	6·0	5·2	5·2
	0·1	6·6	6·4	6·2	6·4	6·2	5·2
LA + lactose	0·4	5·8	5·4	5·2	5·2	5·2	5·2
	0·2	6·2	5·8	5·4	6·0	5·2	5·2
	0·1	6·4	6·2	6·0	6·4	6·2	5·2
LA + maltose	0·4	5·2	5·2	5·2	5·2	5·2	5·2
	0·2	6·2	5·4	5·2	6·2	5·2	5·2
	0·1	6·4	6·2	5·8	6·4	6·4	5·2
LA + mannitol	0·4	5·4	5·2	5·2	5·2	5·2	5·2
	0·2	6·2	5·8	5·2	6·2	5·2	5·2
	0·1	6·4	6·2	6·0	6·4	6·4	5·2

Table 6. *Effect of growth medium on* pH *value attained by* Klebsiella aerogenes *NCTC* 243 *in* 24 *hr. at optimal glucose : buffer ratio*

	Medium on which culture was grown		
Glucose concentration (M)	LA + KNO_3	LA + KNO_3 + glucose	GA
	pH value		
0·4	5·0	6·2	6·2
0·2	5·0	6·2	6·2
0·1	5·4	6·2	6·2

time and temperature standards. Clark & Lubs (1915) used 30°, incubated cultures for at least 3 days and recommended 5 days; in England the Ministry of Health method (Report, 1939) specified 3 days at 37°.

The indicator used has always been methyl red, but the critical range between 5·2 and 5·8 also lies at the acid end of the bromcresol purple range.

Bromcresol purple has a distinct advantage over methyl red: it can be included in the microtest as an internal indicator and readings can be taken at any time; methyl red is rapidly decolorized by many coliform bacteria and can only be used at the conclusion of the test.

During the fermentation of glucose by bacterial cells the hydrogen-ion concentration depends primarily on the glucose : buffer ratio; for coliform bacteria in an excess of glucose the final pH value will be below 5, but in excess of buffer it will be above 6. At the optimal glucose : buffer ratio suspensions of *Bact. coli* and *K. aerogenes* have different and distinctive effects; after 24 hr. *Bact. coli* shows a pH value of 5·0 or less, *K. aerogenes* 5·8 or more. These differences are not seen with cell suspensions from glucose-free media; glucose adaptation seems to be essential for a successful test of this type. The optimum glucose : buffer ratio generally applies between 0·1 and 0·8 M-glucose and 0·025 and 0·2 M-phosphate buffer, but a few strains of *Bact. coli* produced anomalous results with the lowest concentrations. Consequently, as a test for the characterization of coliform bacteria 0·2 M-glucose and 0·05 M-phosphate buffer or 0·4 M-glucose and 0·1 M-phosphate buffer are recommended; these concentrations maintain the optimal glucose : buffer ratio and give reasonably consistent results.

The translation of pH values into the MR test is largely arbitrary; at the extremes there is no difficulty in fixing positive and negative values; I have used pH 5·0 as MR+ and pH 5·8 as MR−, with a doubtful or ± range between these values. Harold (1936), on the other hand, suggested that the line of demarcation between MR+ and MR− should be 5·8; at 5·8 and above the test is negative and at 5·6 and below it is positive. The advantage of eliminating the ± or doubtful zone is obvious, but without it repeated tests may suggest an instability that is much less obvious with an intermediate zone.

The microtest developed is not readily applicable to routine work; unlike our other micromethods a degree of precision in the measurement of volumes is essential if the suspension : glucose : buffer ratios are to be maintained. A very dense suspension is necessary; suspensions adequate for other microtests may give irregular results in the MR. The usual MR test on glucose peptone broth cultures is not without its difficulties, many of which can be overcome by the use of defined media (Clark & Lubs, 1917; Richards & Fouad, 1952). Suspensions in place of growing cultures, and glucose instead of a glucose-peptone mixture, simplify the conditions of the test but they do not remove all the sources of irregularity.

I wish to thank all those who supplied me with cultures of coliform bacteria, especially Drs Kauffmann, Richards and Windle Taylor.

REFERENCES

Clark, W. M. & Lubs, H. A. (1915). The differentiation of bacteria of the colon-aerogenes family by the use of indicators. *J. infect. Dis.* **17**, 160.

Clark, W. M. & Lubs, H. A. (1917). Improved chemical methods for differentiating bacteria of the coli-aerogenes family. *J. biol. Chem.* **30**, 209.

Clarke, P. H. & Cowan, S. T. (1952). Biochemical methods for bacteriology. *J. gen. Microbiol.* 6, 187.

Cowan, S. T. (1953). Fermentations: biochemical micromethods for bacteriology. *J. gen. Microbiol.* 8, 391.

Harold, C. H. H. (1936). *Rep. metrop. Wat. Bd.* No. 30, pp. 97–8.

Hoogerheide, J. C. (1940). Studies on capsule formation. II. The influence of electrolytes on capsule formation by *Klebsiella pneumoniae*. *J. Bact.* 39, 649.

Report (1939). The bacteriological examination of water supplies. *Rep. publ. Hlth med. Subj., Lond.* no. 71.

Richards, T. & Fouad, M. T. A. (1952). The use of glucose inorganic media in the classification of coli-aerogenes bacteria. *Proc. Soc. appl. Bact.* **15** (in the Press).

(*Received* 26 *February* 1953)

McLean, S. J. (1953). *J. gen. Microbiol.* 9, 110–118.

Identification of Strains of *Streptococcus pyogenes* of Types 5, 11, 12, 27 and 44 by the Precipitin Test for the T Antigen

By SIBELY J. McLEAN*

Streptococcal Reference Laboratory, Public Health Laboratory Service,
Colindale Avenue, London, N.W. 9

SUMMARY: Strains of *Streptococcus pyogenes* belonging to Types 5, 11, 12, 27 and 44 share a minor T antigen. In addition, there are three major T antigens in this set of types, one in Type 11, one in Type 12 and the third common to Types 5, 27 and 44.

T antigen, prepared by Pakula's method, is a satisfactory fraction for absorbing the antibodies from serum, the advantage being that very little serum is lost with each absorption because the volume of T antigen required is so small. The Pakula method of extraction of the T antigen can also be satisfactorily used in routine typing. Type 11 strains can be definitely identified by the T precipitin test, and this test can also be used for identifying Type 12 strains that give no reaction with Type 12 anti-M sera. There are some strains which have agglutination patterns of the series 5, 11, 12, 27 and 44 but which give no precipitation with the T antisera. It may be that in these the major T antigen has not been recognized.

Streptococci of Lancefield's Group A (*Streptococcus pyogenes*) generally have two type-specific antigens, known as M and T. Lancefield (1940–1) proposed that the M antigen should be regarded as determining the type of a strain because no strain was known to carry more than one M antigen, and also because the M antigen was more closely related to virulence than the T antigen. Moreover, the M antigen could be recognized by the readily standardized micro-precipitin test (Swift, Wilson & Lancefield, 1943). However, it has been a common experience that it is practically impossible to make anti-M precipitating sera for streptococci of a number of the types that Griffith (1934) recognized by slide-agglutination tests, even with strains freshly isolated from patients; strains of such types still have to be recognized by the agglutination method.

Slide-agglutination typing is well known to be complicated by mutual cross-reactions with certain series of types. In two such series—those comprising Types 15, 17, 19, 23 and 30, and Types 4, 24, 26, 28, 29 and 46—the cause of the cross-reactions has been shown to be a sharing of one or more T antigens (Stewart, Lancefield, Wilson & Swift, 1944). However, the type of these strains can be often decided by the M precipitin test since satisfactory anti-M sera for all types, except perhaps Type 4, are available.

Griffith (1934) recognized a cross-reaction between his Types 11 and 12, and in our experience the series of Types 5, 11, 12, 27 and 44 commonly show varying degrees of cross-reaction. With this series, the type of a strain cannot always be determined by the M antigen because we have been unable to prepare satisfactory anti-M sera for Types 11, 27 or 44.

---

* Present address: Department of Bacteriology, University of Adelaide, South Australia.

The present paper reports an investigation of the T antigens responsible for the cross-reactions among Types 5, 11, 12, 27 and 44, in which use has been made of the method devised by Pakula (1951) for the extraction of the T antigen. This investigation led to a means for the recognition of Type 11 strains in routine work by the T-precipitin test.

MATERIALS AND METHODS

Strains. The strains used in the preliminary investigations were:

Type		Type	
5	Dekens (Griffith)	27	SF 40 (Griffith)
11	Blackmore (Griffith)	44	Henson glossy (Lancefield)
12	SF 42 (Griffith)		

For the subsequent investigation of a routine typing method, strains obtained from three sources were used:

(1) Strains sent to this laboratory for type identification.

(2) Strains isolated here during a large scale epidemiological survey of streptococcal infection in children.

(3) A collection of strains sent from the United States as untypable by the M precipitin test.

The strains were selected because they were agglutinated by some or all of the sera for the five types, 5, 11, 12, 27 and 44, and gave no reaction with the available M sera, namely those for Types 5 and 12.

Preparation of antisera. Antisera were prepared by repeated intravenous inoculation of rabbits with vaccines that had been grown at 22° to increase the production of T antigen, digested with pancreatic extract (Cole & Onslow), and killed with formalin. The pancreatic digestion destroyed the M antigen so that anti-T sera were produced. Group antibodies were removed from the serum by absorption with a suspension of Type 6 'glossy' organisms.

Extraction of the T antigen

Preparation of T fraction for use in absorptions. The T antigen was extracted by a slight modification of Pakula's method. Cultures were grown in 2 l. amounts of nutrient broth + 0·5 % glucose for 2 days. Incubation was at 22° to enhance the production of T antigen. The cultures were heated to 80° for 10 min. and then allowed to settle overnight, when the clear supernatant could be siphoned off. The deposit was washed twice with saline and suspended in 21·6 ml. phosphate buffer, pH 7·8; 2·4 ml. pancreatic extract were added, the pH value was brought to 8·2 and the mixture digested at 50° for 2 hr. The supernatant was acidified to pH 2·5 to precipitate the T-containing fraction and left overnight in the cold. The centrifuged deposit from this contained the T antigen in a concentrated form and was the fraction used for the absorptions.

Preparation of T extracts for use in T precipitin tests. Pakula prepared his T extracts from at least 250 ml. of culture, and in some cases found 500 ml. or 1 l.

necessary. Preliminary tests showed, however, that 100 ml. amounts of culture gave satisfactory results if the culture grew well and if the extracted T antigen was precipitated by acid and redissolved in a small quantity of buffer. The method of extraction was that given above, using 2·7 ml. phosphate buffer and 0·3 ml. pancreatic extract. After acid precipitation the deposit was dissolved in 0·1 ml. phosphate buffer pH 7·8. This constituted the T extract, which was used for precipitin tests in capillary tubes (Swift *et al.* 1943).

Absorption of antisera with T antigen

Preliminary absorptions, using Type 12 serum undiluted and diluted 1/2·5 and 1/5 with saline, showed that the serum diluted 1/5 still gave a reasonable precipitate with the homologous T extract and was much more readily absorbed than the undiluted serum. Accordingly, all the absorptions were done with serum diluted 1/5. Phosphate buffer, pH 7·8, was used as diluting agent instead of saline because otherwise repeated absorption with T concentrate at pH 2·5 lowered the pH of the serum and interfered with subsequent testing by precipitation.

Absorptions were carried out using the T precipitate from 250 ml. of culture to 0·5 ml. of diluted serum. The mixture was incubated at 37° for 2 hr. and left overnight in the cold if necessary. After each absorption the serum, together with an unabsorbed control, was tested by two methods: slide agglutination with suspensions of all five types (5, 11, 12, 27, 44) and precipitation with T extracts of each type. Absorption was continued until all the antibodies for the homologous extract were removed. The number of absorptions required varied from one to three with different sera.

Agglutination tests. These were done by the slide method using suspensions grown in buffered broth containing 0·2 % glucose for 2 days at 22°. If any of the suspensions were granular they were treated with pancreatic extract at 50° for 20 min. The suspensions used for titrating the five types of sera and for testing the absorbed sera were similarly treated to destroy the M antigens.

RESULTS

Related T antigens in types 5, 11, 12, 27 *and* 44

Demonstration of the cross-reactions between the five types

Slide-agglutination and T-precipitin tests suggested that the five types have a T antigenic component in common, and that, in addition, Types 5, 27 and 44 share a major T antigen (Table 1).

Pakula (1951) showed the presence of related T antigens in Types 12, 27 and 44; Types 27 and 44 shared a major T antigen as shown by degree of cross-agglutination and by the fact that their T extracts precipitated well with sera of both types. Type 12 had a major T antigen of its own, as it only agglutinated to low titres with sera of Types 27 and 44 and its T extract did not precipitate with these sera. The relationship is confirmed by the results in Table 1, which also indicate that Type 11, like Type 12, has a major T antigen of its own, whereas Type 5 shares the T antigen of Types 27 and 44.

The relationship of the T antigens as shown by reciprocal absorptions

Preliminary work with Type 12 serum showed that absorption of the T antibody with a suspension of Type 12 organism sometimes required as many as five or six absorptions, and this led to the loss of an appreciable amount of serum. The use of a concentrated T antigen extracted by Pakula's method proved more effective in absorption and led to little loss of serum.

Table 1. *Agglutination- and T-precipitin reactions showing that Types* 5, 11, 12, 27 *and* 44 *have related T antigens*

Antisera	Temperature of incubation for growth of culture (°)	Reactions in agglutination and precipitin tests with strains of Types									
		5		11		12		27		44	
		Agg.	Ppt.	Agg.	Ppt.	Agg.	Ppt.	Agg.	Ppt.	Agg.	Ppt.
Type 5	22	160	+++	5	–	5	–	320	++++	320	++++
	37	1	.	10	.	–	.	160	.	320	.
Type 11	22	–	–	160	+++	–	–	1	–	–	–
	37	–	.	160	.	–	.	1	.	–	.
Type 12	22	1	–	1	–	160	+++	5	–	–	–
	37	–	.	1	.	160	.	20	.	5	.
Type 27	22	320	+++	5	–	1	–	320	++++	320	++++
	37	–	.	5	.	1	.	320	.	320	.
Type 44	22	160	+++	–	–	–	–	320	++++	160	++++
	37	–	.	–	.	–	.	320	.	160	.

The figures are the reciprocal of the highest dilution at which agglutination (Agg.) still occurred; – = no agglutination. Decreasing strength of precipitation (Ppt.) indicated by the symbols ++++, +++, ++, +, ±; – = no precipitation. Precipitation results read after incubation at 37° for 2 hr. and refrigeration overnight.

Antisera for each of the five types were first absorbed with Type 6 'glossy' organisms to remove group antibodies. Portions of each serum were then absorbed with T antigen from each type until all the antibodies for the type used for absorption had been removed. Absorbed sera were tested both by slide agglutination and T precipitation with suspensions or extracts of all five types (Table 2). The strength of the precipitin reactions in this table is generally less than in Table 1 as the sera used in these experiments were diluted 1/5.

Type 11 has a distinct T antigen and its agglutinins and precipitins were not removed from the antiserum by absorption with the other four types although they were decreased, possibly because the serum required three absorptions. Type 11 also only absorbed the agglutinins for itself from the sera for Types 5, 27 and 44. In the same way Type 12 has a distinct T antigen. Types 5, 27 and 44 each removed the agglutinins and precipitins from the sera for Types 5, 27 and 44, although Type 5 did not remove all the agglutinins from the sera of Types 27 and 44, probably because the absorbing extracts were not as strong as those of 27 and 44.

It appears, therefore, that there are three major T antigens in this set of five strains, one in the Type 11 strain, another in the Type 12 and a third in the strains of Types 5, 27 and 44. Type 5 produces little or none of this antigen when grown at 37°, and when grown at 22° usually produces less than Types 27 and 44 as shown by the amount extracted by Pakula's method.

Table 2. *Reciprocal absorptions showing relationship of T antigens of Types* 5, 11, 12, 27 *and* 44

	Reactions in agglutination and precipitin tests with strains of Types									
	5		11		12		27		44	
Antisera diluted 1:5	Agg.	Ppt.	Agg.	Ppt.	Agg.	Ppt.	Agg.	Ppt.	Agg.	Ppt.
Type 5 serum										
Unabsorbed	320	++	5	–	5	–	320	+++	640	++
Absorbed with Type 5	–	–	–	–	–	–	–	–	–	–
Absorbed with Type 11	160	++	–	–	–	–	160	+++	640	++
Absorbed with Type 12	160	+	–	–	–	–	160	+++	640	++
Absorbed with Type 27	–	–	–	–	–	–	–	–	–	–
Absorbed with Type 44	–	–	–	–	–	–	–	–	–	–
Type 11 serum										
Unabsorbed	–	–	160	++	–	–	–	–	–	–
Absorbed with Type 5	–	–	20	++	–	–	–	–	–	–
Absorbed with Type 11	–	–	–	–	–	–	–	–	–	–
Absorbed with Type 12	–	–	20	+	–	–	–	–	–	–
Absorbed with Type 27	–	–	20	++	–	–	–	–	–	–
Absorbed with Type 44	–	–	40	++	–	–	–	–	–	–
Type 12 serum										
Unabsorbed	–	–	–	–	320	++	5	–	5	–
Absorbed with Type 5	–	–	–	–	160	+	–	–	–	–
Absorbed with Type 11	–	–	–	–	160	+	–	–	–	–
Absorbed with Type 12	–	–	–	–	–	–	–	–	–	–
Absorbed with Type 27	–	–	–	–	160	+	–	–	–	–
Absorbed with Type 44	–	–	–	–	160	+	–	–	–	–
Type 27 serum										
Unabsorbed	160	++	5	–	5	–	320	+++	640	++
Absorbed with Type 5	–	–	–	–	–	–	5	–	10	–
Absorbed with Type 11	160	++	–	–	–	–	320	++	640	++
Absorbed with Type 12	160	+	–	–	–	–	160	++	640	++
Absorbed with Type 27	–	–	–	–	–	–	–	–	–	–
Absorbed with Type 44	–	–	–	–	–	–	–	–	–	–
Type 44 serum										
Unabsorbed	160	+++	5	–	5	–	320	+++	320	++
Absorbed with Type 5	10	–	–	–	–	–	20	–	20	–
Absorbed with Type 11	160	++	–	–	–	–	320	+	320	+
Absorbed with Type 12	160	++	–	–	–	–	160	++	320	+
Absorbed with Type 27	–	–	–	–	–	–	–	–	–	–
Absorbed with Type 44	–	–	–	–	–	–	–	–	–	–

Conventions as in Table 1.

These results also show that the T antigen fraction is satisfactory for use in serum absorption; it is largely dissolved in the diluted serum and any precipitate that forms can be readily removed by centrifugation. Very little serum is lost with each successive absorption.

Routine identification of Type 11 *strains by the T-precipitation test*

For routine typing of streptococci it is desirable to have a method in which the unknown strain reacts with the serum of only one type. This is not always possible with the slide-agglutination method because of cross-reactions due to the sharing of T antigens by certain types. The alternative Lancefield M precipitation method is also impracticable for some types, such as 11, 27, and 44, because of the difficulty of preparing M antisera.

This paper has shown that it is possible to distinguish Type 11 from Types 5, 12, 27 and 44 by the T-precipitin test, precipitation being obtained only with the homologous serum. Pakula found that T extracts gave precipitation with sera with agglutinating titres of 1/160 or more. Type 11 strains agglutinate with sera of Types 5, 12, 27 and 44 to low titre so that the result from agglutination tests with sera at low dilution is a 'pattern', but these heterologous reactions are eliminated by the T-precipitin test. Further investigation was therefore carried out to test whether T precipitation was of value in routine typing, with special reference to the identification of Type 11 strains.

As a preliminary trial all our stock strains of Types 5, 11, 12, 27 and 44 were tested by agglutination and T precipitation (Table 3). Type 5, 27 and 44 organisms had agglutination patterns with strong reactions with the sera for Types 5, 27 and 44, as would be suspected from the results of the previous work. Type 11 organisms agglutinated strongly with Type 11 serum and also agglutinated with Type 27 and 44 sera, while Type 12 organisms agglutinated with Type 12, 27 and 44 sera. The T-precipitin tests agreed well with the agglutinations.

The method of extraction having proved satisfactory with these stock strains, seventy-six new strains were then tested in a similar manner (Table 4). The 'original agglutination patterns' were those given when the strain was first received in the laboratory and the 'repeat agglutination patterns' were those given when the strains were re-tested, sometimes several months later. The agglutination patterns, and the strength of the individual reactions in each pattern, remained fairly constant with strains that gave a positive T-precipitin reaction, but were more variable with the strains that failed to precipitate with any of the five sera. Using the results of Table 3, the type of each new strain could be inferred from the strength of the reactions in its agglutination pattern.

There were twenty-six strains having agglutination patterns which did not fit into any of those in Table 3, and none of these strains gave any reaction in the T-precipitin test. When strains gave a negative reaction, the extraction and precipitation were repeated, and if the results were still negative the extraction was repeated again from 250 ml. of culture, before being finally regarded as negative.

There are two possible explanations of the failure of these strains to precipitate with the T sera. Since they have agglutination patterns there must presumably be some T antigens present. Therefore, either the method of extraction is not suitable, which seems unlikely in view of the number of strains that do give positive results, or these agglutinations with Types 5, 11, 12, 27 and 44 are minor reactions, and the major T antigens of these strains are not yet identified. If this were so, these strains should only agglutinate to low titres with the five antisera. Several of these negative strains and several strains giving positive results in the T-precipitin test were titrated against the five sera (Table 5). The strains giving positive results by T precipitation agglutinated to high titres with the type serum with which they precipitated, but the strains giving no results by T precipitation did not agglutinate to high titre with any of the five types. The agglutination patterns of these strains are therefore probably due to minor T antigens and the major T antigens are not yet identified. Indeed these strains could be new types. The presence of such strains, which share minor T antigens with the Types 5, 11, 12, 27 and 44,

Table 3. *Agglutination patterns and T-precipitation results of stock strains*

			T precipitation. Antisera				
Strain	Source	Agglutination pattern	Type 5	Type 11	Type 12	Type 27	Type 44
Type 5							
Dekens	Griffith	5^{++} 27^{++} 44^{++}	+	–	–	+	+
Veen	Griffith	5^{++} 27^{++} 44^{++}	+ +	–	–	+ +	+ +
T5 B	Lancefield	5^{++} 27^{++} 44^{++}	+ +	–	–	+ +	+
R51.479	Routine	5^{++} 27^{++} 44^{++}	+	–	–	+ +	+
R51.776	Routine	5^{++} 27^{++} 44^{++}	+	–	–	+	+
B8100	Routine	5^{++} $12^{\pm}$ 27^{++} 44^{++}	+	–	–	+ +	±
Type 11							
Adams	Griffith	11^{++} 12^{+} 44^{++}	–	+ + +	–	–	–
Bethell	Griffith	11^{++} 27^{++} 44^{++}	–	+ +	–	–	–
Blackmore	Griffith	11^{++} 44^{++}	–	+ +	–	–	–
Curtis	Griffith	11^{++} 27^{++} 44^{++}	–	+ +	–	–	–
Dobson	Griffith	11^{++} 27^{+} 44^{++}	–	+ + +	–	–	–
T11	Lancefield	11^{++} 27^{+} 44^{++}	–	+ +	–	–	–
Type 12							
SF42	Griffith	12^{++} $27^{\pm}$ 44^{++}	–	–	+ +	–	–
869	Routine	12^{++} 27^{+} 44^{++}	–	–	+ +	–	–
R47.3722/17 P.S.	Routine	12^{++} 44^{+}	–	–	+ +	–	–
R.1518	Routine	12^{++} 44^{++}	–	–	+ +	–	–
R50.41	Routine	12^{++} 44^{+}	–	–	+ +	–	–
R51.178	Routine	12^{++} 44^{+}	–	–	+ +	–	–
B 8400	Routine	12^{++} 44^{++}	–	–	+ +	–	–
Type 27							
Makinson	Griffith	5^{++} 11^{+} 12^{+} 27^{++} 44^{++}	+ + + +	–	–	+ + + +	+ + +
Prince Smith	Griffith	5^{++} 11^{+} 27^{++} 44^{++}	+	–	–	+	+
Q49 T	Griffith	5^{++} 11^{+} 12^{+} 27^{++} 44^{++}	+ + +	–	–	+ + +	+ + +
ERY	Griffith	5^{++} $11^{\pm}$ $12^{\pm}$ 27^{++} 44^{++}	+ +	–	–	+ +	+ +
SF40	Griffith	5^{++} 27^{++} 44^{++}	+ +	–	–	+ +	+ +
T27/46	Lancefield	5^{++} 11^{+} 12^{+} 27^{++} 44^{++}	+ + +	–	–	+ + +	+ + +
T27 A/32	Lancefield	5^{++} $11^{\pm}$ 12^{+} 27^{++} 44^{++}	+ + +	–	–	+ + +	+ + +
Type 44							
A402EG18	Griffith	5^{++} 27^{++} 44^{++}	+ +	–	–	+ +	+ +
Henson	Lancefield	5^{++} $12^{\pm}$ 27^{++} 44^{++}	+	–	–	+	+

Conventions as in Table 1.

confirms the usefulness of the T-precipitin test for the positive identification of these five types.

Of the seventy-six new strains that gave no reactions with Type 5 or Type 12 anti-M sera, fifty reacted in the T-precipitin test. Of these fifty strains, twenty-six reacted with the Type 11 serum, twelve with Type 12, and the remaining twelve with the sera for Types 5, 27 and 44. As a method of identifying Type 11 strains the T-precipitin test seems very satisfactory. The type 12 strains gave no M precipitation and apart from the T-precipitin test could not have been definitely identified.

Table 4. *Summary of agglutination patterns and T-precipitation results of new strains*

T precipitation	Original agglutination pattern	Repeat agglutination pattern	No. of strains
5, 27, 44	5^{++} 11^{+} 12^{+} 27^{++} 44^{++}	5^{++} 11^{+} 12^{+} 27^{++} 44^{++}	12
	5^{++} 12^{+} 27^{++} 44^{++}	5^{++} 12^{+} 27^{++} 44^{++}	
	5^{++} 27^{++} 44^{++}	5^{++} 27^{++} 44^{++}	
11	5^{+} 11^{++} 27^{+} 44^{++}	5^{+} 11^{++} 27^{++} 44^{++}	26
	11^{++} 27^{++} 44^{++}	11^{++} 27^{++} 44^{++}	
	11^{++} 44^{++}	11^{++} 44^{++}	
12	11^{+} 12^{++} 27^{+} 44^{++}	12^{++} 27^{+} 44^{++}	12
	12^{++} 27^{++} 44^{++}	12^{++} 44^{++}	
	12^{++} 44^{++}	12^{++}	
	12^{++}		
–	5^{++} 11^{+} 27^{+} 44^{++}	5^{++} 11^{+} 27^{+} 44^{++}	26
	5^{+} 11^{+} 27^{+} 44^{+}	5^{+} 11^{+} 27^{++} 44^{++}	
	5^{+} 11^{+} 44^{++}	5^{+} 11^{+} 44^{++}	
	12^{+} 27^{+} 44^{++}	11^{+} 12^{+} 44^{++}	
	11^{+} 27^{+} 44^{+}	11^{+} 44^{++}	
	11^{+} 27^{+} 44^{++}	11^{+} 44^{+}	
	27^{+} 44^{++}	44^{+}	
	44^{++}		

Conventions as in Table 1.

Table 5. *Agglutination titres of groups of strains giving positive and negative results by T precipitation*

		Agglutination titre with sera of types				
Strain	Type by T precipitation	5	11	12	27	44
Rz 15	5, 27, 44	640	.	5	320	320
22	11	20	160	1	1	10
31	12	20	.	640	5	80
40	5, 27, 44	1280	.	1	320	320
58	11	10	80	.	10	10
18	.	5	.	1	1	10
23	.	20	.	5	10	20
25	.	40	10	5	10	10
46	.	10	.	5	1	10
47	.	20	.	1	5	10

Conventions as in Table 1.

Since the T-precipitin test only serves to eliminate the minor cross-reactions, the same result should be obtained by slide-agglutination tests using sera suitably diluted to remove the cross-reactions. All the new strains were retested by slide agglutination using sera diluted 1/80. As a rule the results were the same as those from the T-precipitin tests, but with some strains weak cross-reactions still occurred. To type strains with the use of diluted serum would evidently need careful preliminary titration of the serum. The T-precipitin test, however, eliminates these minor cross-reactions in all cases and so seems the method of choice.

DISCUSSION

Lancefield defined the type of Group A streptococci by the M-antigens because these were not shared by different types and could readily be detected in acid extracts by the capillary precipitin technique. It is now generally conceded that this is the method of choice for type identification of streptococci. However the M-precipitin test requires satisfactory anti-M sera, and it has been our experience that sera for some types may react with the vaccine strain but with none of a number of other strains that are judged by slide agglutination to belong to the same type. For the type identification of such strains, for which there are no satisfactory anti-M sera, we have to rely on the slide-agglutination test, and this is complicated by cross-reactions between certain types due to sharing of T antigens.

The present work suggests that the T-precipitin test may be useful for the identification of strains, such as those of Types 11, 27 and 44, for which anti-M sera are not readily prepared, and which normally give a 'pattern' reaction in slide-agglutination tests owing to their sharing of T antigens.

For routine type identification, a preliminary slide-agglutination test, followed by the M-precipitin test for confirmation, seems desirable. Usually this is satisfactory but when no M sera are available the T-precipitin test can be adapted for the routine identification of many strains within the series 5, 11, 12, 27 and 44.

REFERENCES

GRIFFITH, F. (1934). The serological classification of *Streptococcus pyogenes*. *J. Hyg., Camb.* **34**, 542.

LANCEFIELD, R. C. (1940–1). Specific relationship of cell composition to biological activity of hemolytic streptococci. *Harvey Lect.* **36**, 251.

PAKULA, R. (1951). Extraction of the T antigen of *Streptococcus pyogenes*. *J. gen. Microbiol.* **5**, 640.

STEWART, W. A., LANCEFIELD, R. C., WILSON, A. T. & SWIFT, H. F. (1944). Studies on the antigenic composition of Group A hemolytic streptococci. IV. Related T but distinct M antigens in Types 15, 17, 19, 23, 30 and in Types 4, 24, 26, 28, 29, 46. Identification by slide agglutination. *J. exp. Med.* **79**, 99.

SWIFT, H. F., WILSON, A. T. & LANCEFIELD, R. C. (1943). Typing Group A hemolytic streptococci by M precipitin reactions in capillary pipettes. *J. exp. Med.* **78**, 127.

(*Received* 2 *March* 1953)

FULTON, F. & ISAACS, A. (1953). *J. gen. Microbiol.* 9, 119–131.

Influenza Virus Multiplication in the Chick Chorio-allantoic Membrane

BY F. FULTON
The London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C. 1
AND A. ISAACS
The National Institute for Medical Research, Mill Hill, London, N.W. 7

SUMMARY: A description is given of techniques used for studying influenza virus infection of the allantoic and chorionic surfaces of the chick chorio-allantoic membrane. It was possible to infect one surface of the membrane at a time and to show that the two layers behaved independently and reacted differently. In contrast to the allantoic layer, the chorionic cells appeared to support only a single cycle of multiplication. This conclusion is based particularly on the small rise of infectivity which occurs following chorionic inoculation of virus, and on the proportion between the amount of virus inoculated and the yield of soluble antigen. Some evidence is presented which suggests that the elementary bodies are less readily released from chorionic cells than from the allantoic cells.

In a preliminary communication (Isaacs & Fulton, 1953), it has been shown that there is an essential difference in response of chorionic and allantoic cells of the chick chorio-allantoic membrane to infection with influenza A virus. The purpose of this paper is to present a detailed analysis of this difference and to describe the special techniques devised to study the problem.

MATERIALS AND METHODS

THE DESIGN OF APPARATUS FOR CULTIVATING INFLUENZA VIRUS IN EXCISED CHORIO-ALLANTOIC MEMBRANE

Preliminary experiments *in ovo* had shown that influenza virus A inoculated on to the chorionic surface of the chorio-allantoic membrane appeared to undergo a development which could best be explained by supposing that virus multiplication was strictly confined to the surface inoculated, and that the virus only went through one cycle of multiplication, after which the process came to a stop, presumably because of the failure of the cells first infected to liberate new virus. This contrasted notably with the sequence of events which followed inoculation of the virus into the allantoic cavity. As is well known, the allantoic cells become infected, and if the inoculum is not sufficient to saturate all the available cells immediately, the first cycle of multiplication in the infected cells is followed by the liberation into the allantoic cavity of new virus, which is then adsorbed on to still undamaged cells to repeat the process. In this way virus multiplication in the allantoic cells proceeds by a number of cycles until all the available cells have been exhausted.

It was immediately obvious that the different behaviour of the chorionic cells could be due simply to the different physiological conditions on their side of the membrane and need not reflect any fundamental difference in the response of the cells on the two sides to infection. To resolve this problem it was necessary to infect the two sides of the membrane under comparable conditions.

A method of titrating influenza virus in small pieces of excised chorio-allantoic membrane has already been described (Fulton & Armitage, 1951; Fulton, 1952). In this technique the pieces of membrane float in a simple salt solution contained in cavities drilled in a thick block of Perspex; virus is added to the solution and is at liberty to enter cells on either side of the membrane. Two new pieces of apparatus were therefore designed to allow infection to occur on one or other side or on both sides simultaneously under identical physiological conditions.

Apparatus permitting infection of one side of the membrane or of both sides simultaneously

The device which will be referred to as a doublet is constructed of Perspex and is illustrated in Pl. 1. A piece of excised chorio-allantoic membrane is held as a diaphragm between two identical chambers which can be filled with fluid containing virus. The exact dimensions are not of critical importance, but in the model shown the internal diameter of the end chambers is 19 mm. The membrane is held firmly between the two thin Perspex plates which are drilled with four central holes of diameter 6·35 mm. These holes permit fluid to flow freely to the surface of the membrane, but the membrane is supported and prevented from billowing into the adjacent chamber. The central component is a piece of polythene 0·25 mm. thick which prevents the membrane being crushed between the two drilled plates. The inner face of each end chamber is fitted with a circular rubber washer which fits into a circular groove. The washer lies 1 mm. above the surface of the inner face plate and, when assembled, provides an effective fluid seal between the end chambers and the drilled plates.

To assemble the apparatus, the end chamber which holds the four bolts is laid flat on the bench with the inner face plate uppermost. This chamber is engraved with an A and will be on the allantoic side of the membrane. One of the drilled plates is now fitted into position over the bolts, and on it is laid flat a piece of chorio-allantoic membrane with the chorionic side uppermost. It is important to use membranes from 15- to 16-day embryos, as before this time they are too fragile and are liable to be torn and perforated. With a little practice it is easy, by inspection, to distinguish the two sides of the membrane. When the membrane is being removed from the egg a small piece of shell membrane is left adherent to the chorionic side, and as an additional precaution, the allantoic side may be recognized by observing that the large blood vessels are very superficial on this surface.

Before being placed in position on the drilled plate, the excised membrane is freed of shell membrane, washed in glucosol (Parker, 1938) and cut to a suitable size. When in position it is immediately covered with the polythene component, the other drilled plate and finally the second end-chamber which is engraved with a C. The components are then sealed together by means of nuts which screw down on the four bolts. As with all apparatus made of this plastic the tolerances of the holes which fit over the bolts should be generous, to allow for the high coefficient of thermal expansion. It will be observed from the photograph that each end-chamber is pierced laterally by a small hole; since in assembling the apparatus it is important that the holes in the two end-chambers should lie in the same plane, all the Perspex components are notched along one face so that they may be correctly aligned. After assembly, the apparatus is laid horizontal on the bench with the end-chamber holes uppermost. Fluid with or without virus may now be introduced into the chambers through the small holes by means of a fine Pasteur pipette. The volume normally introduced is 5 ml. and, after filling, the chamber is sealed by closing the small holes with a piece of Sellotape (Adhesive Tapes Ltd., Boreham Wood).

The apparatus is now placed in an incubator at 37° for 24 or 48 hr. and kept in constant motion on a rocking machine of the tilting tray type, the tilt being in the long axis of the apparatus. The rocking machine has already been illustrated in an earlier paper (Fulton & Armitage, 1951); in this application, however, it is geared down to give only six sweeps/min. The fluid used in the end-chambers is the modified glucosol with penicillin and streptomycin also described in the earlier paper. At the end of the experiment the fluid is removed by pipette and assayed for haemagglutinin.

Before use, the apparatus is sterilized by immersing it for half an hour in 10 % HCl. It is then washed in sterile distilled water and dried in an incubator maintained at 37°. The components lie on sterile blotting-paper, and precautions are taken to avoid dust falling on them while drying. They are stored in a sterile tin.

Apparatus permitting infection of one side of the membrane only

The device which will be referred to as a unit, was also made of Perspex, and is illustrated in Pl. 2.

The base plate containing the four bolts is 78 mm. square. To assemble it, the base plate is covered with a rubber mat 3 mm. thick. Over the central part of the mat is placed a 40 mm. square of filter-paper which has been soaked in glucosol; over it is laid a slightly larger square of sterile cellophan also soaked in glucosol. A piece of chorio-allantoic membrane is now laid out flat on the cellophan. As with the doublet, it is removed from a chick embryo 15–16 days old, washed in glucosol, carefully orientated and then cut to an appropriate size. On it is placed the contact plate 6 mm. thick and drilled centrally with four holes of diameter 14·3 mm., and over this the spacer plate and the central circular cover to prevent dust falling on the membrane. The components are brought into firm apposition by tightening thumb screws on the four corner bolts. In this way four cells are created which have for a floor a piece of chorio-allantoic membrane, arranged with either side uppermost. The use of four small holes in the central area rather than one large hole was the result of bitter experience; for it proved very difficult to avoid, over so large an area, a small tear which would completely nullify an experiment. With four cells it was usual to obtain at least three in which, at the end of the experiment, the membrane on the floor was seen to be intact.

For certain experiments a nine-hole face plate was used, each hole having a diameter of 8·7 mm. After assembly the cells are filled with an appropriate amount of modified glucosol containing antibiotics and the virus inoculum. In the unit with the four-hole face plate each cell contains 0·25 ml.; in the unit with the nine-hole face plate each cell contains 0·20 ml. The top disk is sealed by running hot wax around its rim, and the unit is rocked for 24 or 48 hr. in an incubator maintained at 37°. The rocking machine is geared to provide forty-five sweeps/min. At the end of the experiment the fluid in each cell is removed and assayed for haemagglutinin; also the intact membranes forming the floors of the cells are cut out with a sharp knife, pooled, ground in a micro Ten Broeck grinder and assayed for complement-fixing antigens.

In preparing the unit for an experiment it is not necessary to sterilize the base-plate, the rubber mat or the filter-paper. The cellophan is sterilized by boiling in distilled water. The other components are soaked for half an hour in 10 % HCl, rinsed in sterile distilled water and then dried in an incubator maintained at 37°.

THE ASSAY OF COMPLEMENT-FIXING ANTIGENS

Suspensions of chorio-allantoic membrane infected with influenza virus contain a mixture of two apparently distinct complement-fixing antigens; the elementary body antigen which is strain specific, and an antigen of much smaller particle size, often referred to as the soluble antigen which is only group specific (Hoyle & Fairbrother, 1937).

In this study it was desired to assay each component of the mixture separately. A common method (Hoyle, 1950) is to use a specific immune ferret serum for the measurement of the elementary body antigen, since such sera often contain only traces of antibody capable of reacting with soluble antigen. For the assay of soluble antigen, human convalescent serum is used which contains large amounts of antibody capable of reacting with the soluble antigen. By choosing sera from patients infected with a strain of influenza virus remote antigenically from the strain in the mixture being assayed, it is possible to employ dilutions of the human serum which react strongly with the soluble antigen and not measurably with the elementary body.

In the preliminary experiments *in ovo* which are reported here, only the complement-fixing soluble antigen was measured and the assay was made with a suitable batch of human convalescent serum using a modification of the technique described by Hoyle (1948).

For later experiments and for all the experiments made *in vitro* a more precise method for the assay of complement-fixing soluble antigen was used and the elementary body antigen was also measured. For the assay of soluble antigen the collection of suitable human sera is clearly a matter of some practical difficulty, and one of us (Fulton, 1949) has suggested an alternative method which employs animal convalescent sera. In that paper ferret sera were used which had been specially selected for high content of antibody reacting with soluble antigen. In the present work a more convenient method is used; Ananthanaryan (to be published) has demonstrated that guinea-pigs infected intranasally with a large dose of purified influenza virus elementary bodies regularly develop in 14 days a high antibody titre to both elementary body and soluble antigen, though the strains differ in their power to evoke antibodies for the soluble antigen. In our experience the Barratt (BAR) strain of A′ virus provokes higher titres of antibody for the soluble antigen than the PR8 strain of A virus given in comparable dose, so that by adjustment of the infecting dose sera possessing the desired characteristics may be prepared. For all the complement-fixation tests with the specific guinea-pig sera the plate technique was used (Fulton & Dumbell, 1949). The apparatus described in that paper has been simplified by using Perspex plates ruled with only 100 squares in 10 rows of 10 columns, and spacing the complement dilutions in the columns at log 0·2 intervals. To facilitate removal of the film of condensation which develops on the plates after removal from the cold and from the hot box, a light breeze from a fan is allowed to play over the plates in the frame before the drop of sensitized blood is added and before the results are read.

In the experiments to be described it was necessary to assay membrane suspensions containing elementary bodies of the PR8 strain of influenza A virus and also the associated soluble antigen. Accordingly, two different lots of guinea-pig serum were prepared.

Guinea-pig serum for assay of PR8 soluble antigen

Fulton & Dumbell (1949) have shown that whereas convalescent mouse antiserum prepared by infection with the PR8 strain contains a large number of antibodies capable of reacting in a complement-fixation test with BAR elementary bodies, the converse is not so. Guinea-pigs behave in the same way, so that if they are infected intranasally with the BAR strain of influenza A′ virus they develop high titres of antibody for the BAR elementary bodies, but the sera do not usually fix complement with the PR8 elementary bodies.

Eleven guinea-pigs, 3 months old, were infected intranasally with BAR elementary bodies purified by one cycle of adsorption and elution on chick erythrocytes. Each animal received 768 units of haemagglutinin contained in 0·5 ml. After 14 days they were bled from the heart and separately assayed for complement-fixing antibodies with standardized BAR and PR8 elementary body suspensions. These antigens are standardized to fix 1·2 log units of complement in the presence of excess homologous guinea-pig serum.

It was desirable that the sera should have a high titre when mixed with the BAR elementary body suspension and a relatively low titre with the PR8 elementary body suspension. Of the eleven sera, nine possessed these characteristics, and they were pooled and dried *in vacuo*. A sample of the pooled dried serum was then reconstituted and assayed again for antibodies reacting with the standardized BAR and PR8 elementary body suspensions, and with influenza A soluble antigen. To avoid any danger of cross-reactions with the homologous elementary body antibody, the soluble antigen was prepared from chorio-allantoic membranes infected with the PR8 strain.

Membrane suspensions (20 % v/v) were adsorbed with chick erythrocytes until there was no demonstrable haemagglutinin; they were then centrifuged at 20,000 *g* for 2 hr.

The results of these titrations are shown in Fig. 1 *a*. At dilutions of 1/10 and greater there was no reaction with the standardized PR8 elementary body suspension or with less concentrated suspensions. The points on the curve of fixation with the BAR elementary body suspension represent the log units fixed with the standardized antigen. With the soluble antigen there is a strongly marked zone of inhibition in the region of antigen excess; these zones are illustrated in Fig. 2. Accordingly, a three-dimensional test had to be set up and the points on the curve of fixation with soluble antigen represent the log units of complement fixed maximally at selected serum concentrations.

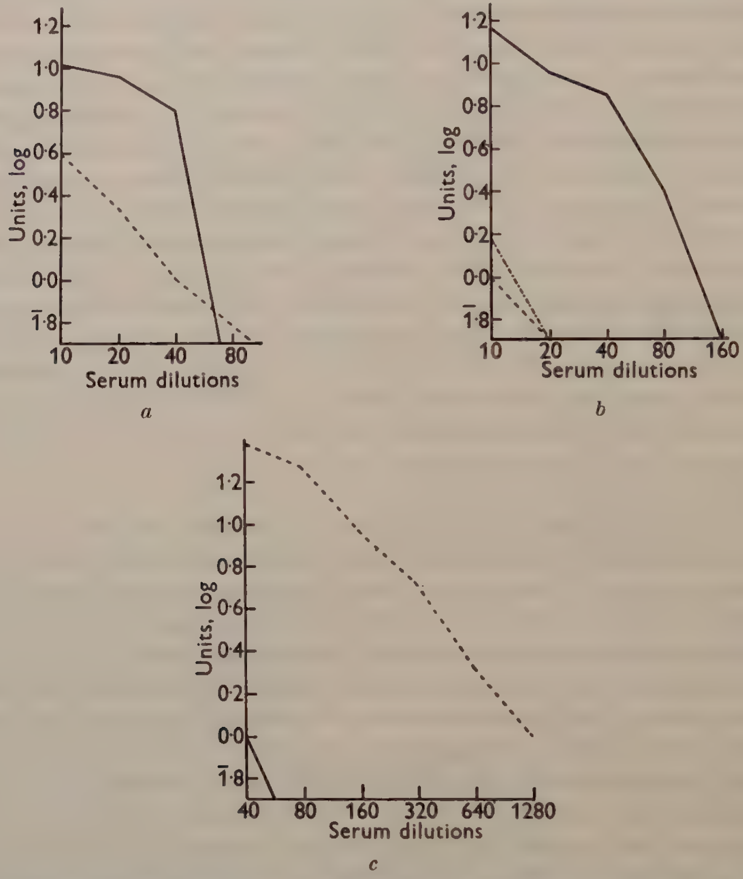


Fig. 1. **Influenza virus complement-fixing antibodies in convalescent sera: log units of complement fixed at selected serum dilutions. (*a*) Guinea-pig immune serum (BAR strain). Solid line: BAR elementary body antigen; broken line: soluble antigen. (*b*) Guinea-pig immune serum (PR8 strain). Solid line: PR8 elementary body antigen; dot-dash line: BAR elementary body antigen; broken line: soluble antigen. (*c*) Human serum. Solid line: PR8 elementary body antigen; broken line: soluble antigen.**

For the specific assay of PR8 soluble antigen the serum is used at a constant dilution of 1/10, and the antigen titre is defined as the highest initial dilution which will fix $\bar{1}{\cdot}7$ log units of complement.

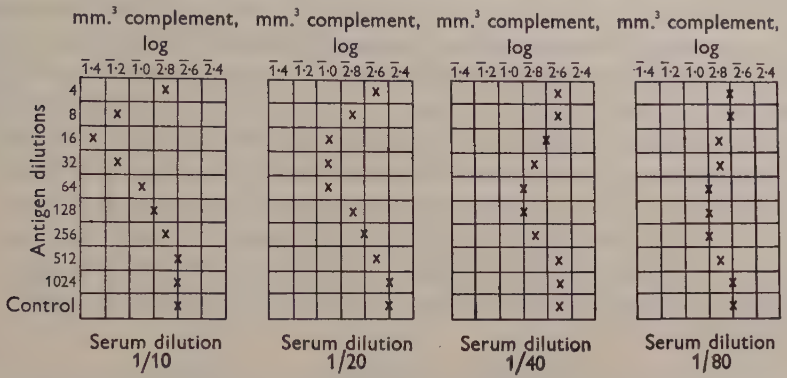


Fig. 2. Complement fixation of PR8 soluble antigen with BAR immune guinea-pig serum diluted 1/10. In each row the cross indicates the column in which the 50 % end-point was observed.

Guinea-pig serum for assay of PR8 elementary body antigen

Ten guinea-pigs, 3 months old, were infected intranasally with PR8 elementary bodies purified by one cycle of adsorption and elution on chick erythrocytes. Each animal received 512 units of haemagglutinin contained in 0·5 ml. After 14 days they were bled from the heart and the sera were separately assayed for complement-fixing antibodies with standardized PR8 and BAR elementary body suspensions. It was desirable that the sera should have high titres when mixed with the PR8 suspension and a relatively low titre when mixed with the BAR suspension. Of the ten sera, six possessed these characteristics and were pooled and dried *in vacuo*. A sample of the pooled dried serum was reconstituted and then assayed again with the standardized PR8 and BAR elementary body suspensions, and with a preparation of BAR soluble antigen. The results of these titrations are shown in Fig. 1*b*. Again the points on the curves of fixation with the two elementary body suspensions represent log units fixed with the standardized antigens. The points on the curve of fixation with the heterologous soluble antigen represent the log units fixed maximally at the selected serum concentrations. For specific assay of PR8 elementary body antigen the serum is used at a dilution of 1/60. The antigen titre is defined as the highest dilution which will fix $\bar{1}{\cdot}7$ log units of complement.

Comparison of the two methods of assaying PR8 soluble antigen

The new method of assaying soluble antigen was compared with the conventional technique. A suitable batch of human serum was obtained, inactivated at 56° for 30 min., and absorbed with 5 % sheep erythrocytes. The serum was then tested using the plate technique with standardized PR8 elementary body antigen, and in a three-dimensional experiment with PR8 soluble antigen. The result is shown in Fig. 1*c*.

Again, in the case of the curve of fixation with soluble antigen the points represent maximal fixations as there is, in dilutions of serum close to the antibody titre, a well-marked zone of inhibition in the region of antigen excess. With standardized PR8 elementary body antigen the antibody titre is 1/64; with the PR8 soluble antigen the antibody titre is approximately 1/1280. At any fixed serum level from 1/80 to 1/1280 the titre of the PR8 soluble antigen preparation was 1/256. Assayed by the guinea-pig serum pool the same soluble antigen preparation also had a titre of 1/256. Thus the two methods are comparable when used with the plate technique. It is, however, impossible to compare directly the titres of an antigen preparation measured with the plate technique and measured with the conventional tube technique at a single complement level, because the conditions of primary fixation are quite different.

MISCELLANEOUS METHODS AND MATERIAL

Strains. Two virus strains were used. The PR8 strain of influenza A virus, and the Barratt (BAR) strain of influenza A′ virus/1947.

Assays of haemagglutinin were made by a pattern test, the unit (A.D.) being the least amount of virus suspension in 0·25 ml. of diluent which, when mixed with an equal volume of 0·5 % chick erythrocytes, caused partial agglutination of the cells.

Membrane suspensions were prepared in one of two ways: (*a*) by grinding in a mortar or Ten Broeck grinder with glass powder; (*b*) by freezing in a bath of alcohol and solid carbon dioxide and thawing slowly, and then repeating the process twice.

Incubation with receptor-destroying enzyme. Before assay of haemagglutinin, all the membrane suspensions were incubated with receptor-destroying enzyme for a sufficient time to destroy inhibitor (Burnet & Stone, 1947).

Infectivity titrations for the experiments *in vitro* were carried out by the tissue suspension method (Fulton & Armitage, 1951). For the experiments made *in ovo*, infectivity titrations were made by conventional methods in eggs.

RESULTS

Experiments carried out in vitro

Assays of haemagglutinin

Using a unit with a nine-hole face plate, a piece of chorio-allantoic membrane was arranged with its allantoic side uppermost. The cells were filled with 0·2 ml. of virus dilution in modified glucosol. The inoculum was $10^{4\cdot3}$ I.D.50's of BAR A′ virus. At this dilution no haemagglutinin is initially demonstrable. After rocking the unit for 48 hr. the fluid in each of the nine cells was separately assayed for haemagglutinin. In two such experiments haemagglutinin was found in sixteen of the eighteen cells in titres up to 1/100.

When the experiments were repeated with the chorionic surface uppermost, in seventeen of the cells there was no demonstrable haemagglutinin; in one of the cells haemagglutinin was present with a titre of 1/10, but in this cell the membrane forming the floor was obviously rent, thus exposing the allantoic side to infection.

Using a doublet with 5 ml. of modified glucosol in each of the end-chambers, BAR virus added in a concentration of $10^{4\cdot3}$ I.D.50's to the allantoic side gave readily demonstrable haemagglutinin in that chamber after 24 or 48 hr., but not in the opposite chamber. When virus was added to the fluid in the chamber on the chorionic side, haemagglutinin was not demonstrable in either chamber after 48 hr. If virus was placed in both chambers, haemagglutinin was demonstrable after 24 or 48 hr. on the allantoic side only.

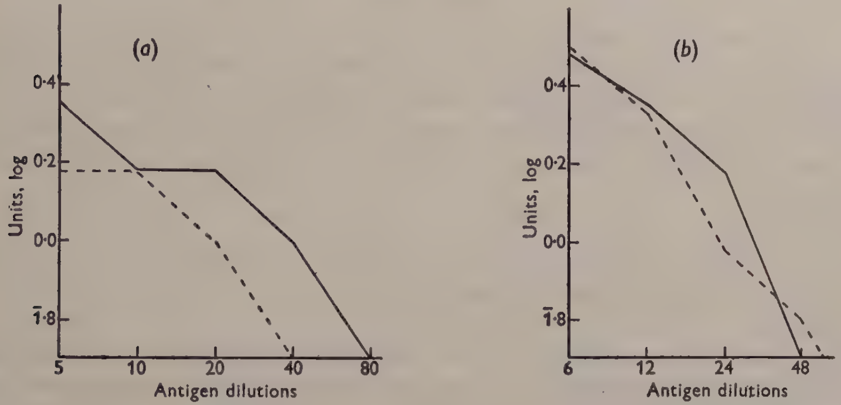


Fig. 3. Complement-fixing antigens in excised chorio-allantoic membrane: log units of complement fixed at selected antigen dilutions. (*a*) Membrane infected on the allantoic surface. (*b*) Membrane infected on the chorionic surface. Solid line: PR8 elementary body antigen; broken line: PR8 soluble antigen.

These results were confirmed on many occasions, and were unaffected even if the sensitivity of the virus assay was enhanced by placing a few pieces of chorio-allantoic membrane floating in the uninoculated fluid on the chorionic side. If amounts of virus too small to be detected by haemagglutination had been released by the chorionic cells, multiplication of the virus would have occurred in the pieces of membrane, and the threshold of the assay would have been attained. From these experiments it is concluded that virus elementary bodies are released into the fluid from the allantoic side in large numbers, but that very little, if any, virus is released from the chorionic side. Virus multiplication seems to be confined to the surface inoculated.

Assay of complement-fixing antigens

The experiments were made with a unit having a four-hole face plate, and the time allowed for virus multiplication was 24 hr. With an inoculum of $10^{4\cdot3}$ I.D.50's of BAR A′ virus, no virus antigens were demonstrable in the cells whose floor was the chorionic surface of the membrane. When the floor of the cell was the allantoic surface, however, virus antigens in high titre were found. An actual titration is illustrated in Fig. 3*a*, where the elementary body antigen has a titre of 1/80, and the soluble antigen a titre of 1/40.

With the chorionic surface uppermost it was possible to demonstrate virus antigens in the membrane after 24 hr. if the virus inoculum was increased to $10^{6 \cdot 3}$ I.D.50's of BAR A′ virus. An actual titration is illustrated in Fig. 3*b*. Both types of antigen were found, the elementary body antigen having a titre of 1/48 and the soluble antigen a titre of 1/60. With these large inocula, there was, of course, demonstrable haemagglutinin in the fluid in the cell at the start of the experiment. But the virus antigens in the membrane do not represent merely seed virus adsorbed to the surface of the membrane, for virus was not found in membranes assayed after incubation for 1 hr., and virus antigens were found after 24 hr. if the inoculum was removed from the cells after 1 hr. and replaced by virus-free diluent.

From these experiments it is concluded that both kinds of virus antigen appear in membranes infected on their chorionic surface, but that a large proportion of the cells must be infected initially if the antigens are to be detected by the relatively insensitive method of assay by complement fixation. This confirms the hypothesis that only a single cycle of virus multiplication is occurring.

Experiments carried out in ovo

The results of inoculating PR8 virus on the chick chorion *in ovo* have been previously described (Isaacs & Fulton, 1953). The main findings from this work are:

(i) Following inoculation on the chorion, PR8 virus did not normally penetrate the chorio-allantoic membrane, but was confined in its growth to the chorionic layer.

(ii) When a small dose of PR8 virus (100 I.D.50's) was inoculated on the chorion a limited increase in infectivity occurred, so that the amount of infective virus increased 10- to 100-fold. No soluble antigen or haemagglutinin was detected in these eggs.

(iii) Following inoculation of a large dose of virus on the chorion there was a rapid production of soluble antigen and, in addition, small amounts of haemagglutinin were detected. The amount of soluble antigen produced was directly related to the amount of virus inoculated, as is shown in Fig. 4. It will also be seen from Fig. 4 that within the limits observed a tenfold increase in the amount of inoculated virus led to a twofold increase in the titre of soluble antigen.

(iv) When a large dose of virus was inoculated on the chorion, the titre of soluble antigen was first detectable after 3 hr. and was high at 24 hr. By 48–72 hr. a considerable decline in titre had occurred. However, this decline in titre could be prevented by giving repeated (twice daily) chorionic inoculations of virus. The results of an experiment which illustrate this are shown in Table 1.

In these experiments only the soluble antigen in the membranes was assayed by complement fixation; virus elementary bodies were measured by assay of haemagglutinin after treatment of the membrane suspensions with receptor-destroying enzyme. The facts that only traces of haemagglutinin were detected in membranes following chorionic inoculation of virus *in ovo*, whereas elementary bodies were readily detected in similar experiments *in vitro* were in apparent contradiction. A number of investigations were carried out on the relative sensitivities of the different techniques used in the preparation of membrane suspensions in the experiments; *in vitro* the membrane suspensions had been made by grinding them in a Ten Broeck grinder, whereas for the experiments *in ovo* the membrane suspensions had been prepared by freezing and thawing. In eggs injected intra-allantoically, similar yields of elementary body and soluble antigen were obtained from membranes

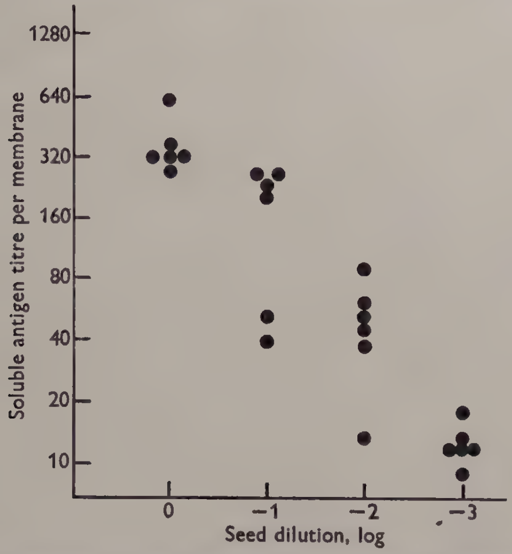


Fig. 4. The relation between the virus inoculum and the titre of complement-fixing soluble antigen which develops when eggs are inoculated on the chorion with dilutions of a PR 8 virus suspension containing $10^{7\cdot8}$ I.D. 50's and $2\cdot5 \times 10^3$ units of haemagglutinin.

Table 1. *Titres of soluble antigen in membranes from thirty eggs at intervals after chorionic inoculation*

	Soluble antigen titre at time (hr.) shown after a single injection				Titre after repeated injections
	6	24	48	72	
	240	640	40	80	1280
	320	640	320	40	640
	20	1280	640	10	640
	120	40	10	160	640
	160	960	50	140	320
	320	960	640	20	640
G.M.	145	513	126	50	640

Inoculum: 0·5 ml. of PR 8 virus containing 1280 units of haemagglutinin.

Membranes from individual eggs were frozen and thawed and titrated for soluble antigen with human serum.

G.M. = geometric mean.

treated either by grinding or by freezing and thawing. In eggs infected on the chorion, both techniques released the same amount of soluble antigen, but more elementary bodies were released by grinding. An experiment illustrating this is shown in Table 2.

Table 2. *Titres of specific antigens in membranes 24 hr. after chorionic infection*

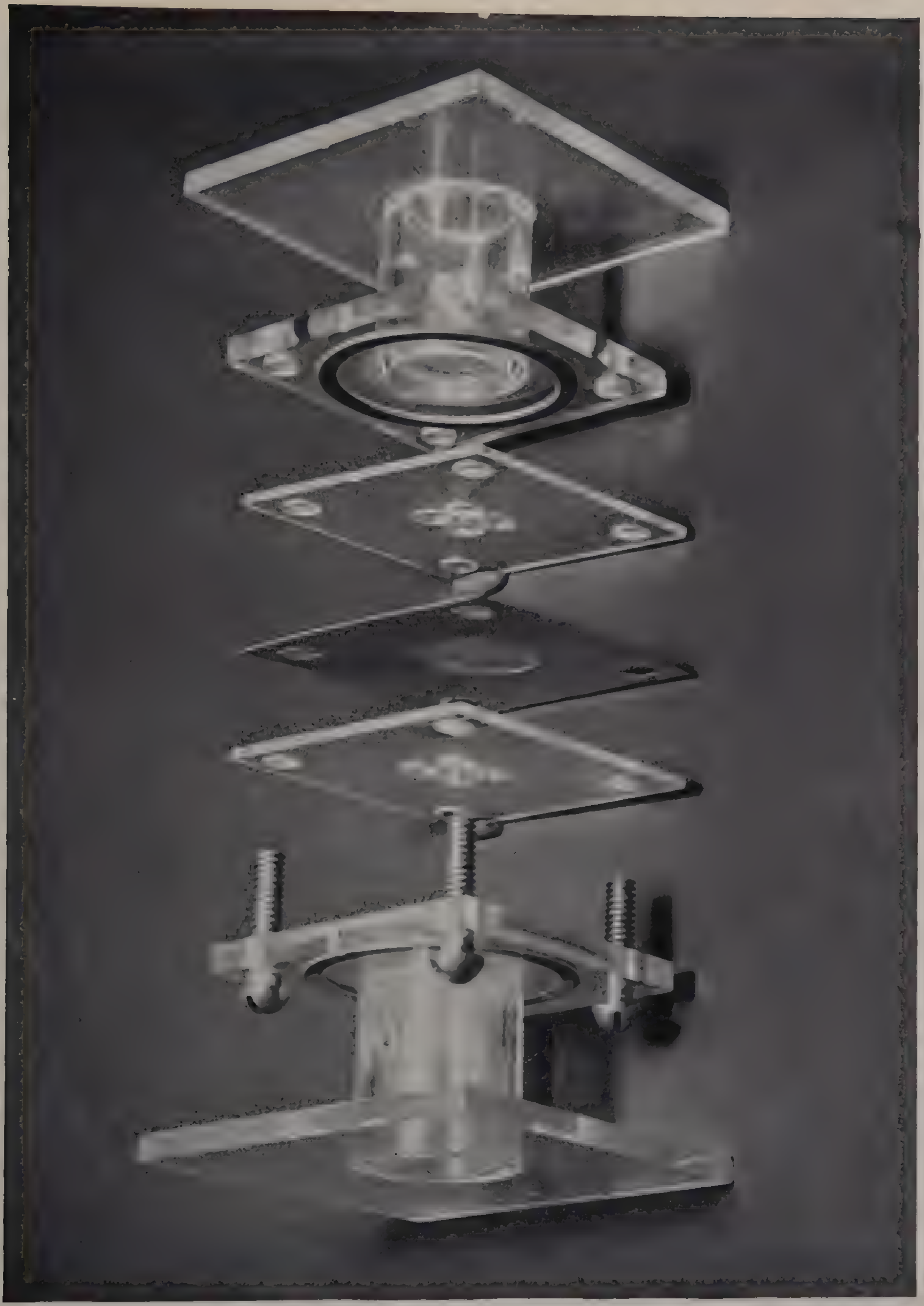
			Haemagglutinin units	Complement-fixing antigen titre 0 = <6	
Egg no.	Area	Treatment	0 = <2	EB*	S.A.†
1	Dropped	Ground	32	7	28
		Frozen	3	0	34
	Residue	Ground	0	0	0
2	Dropped	Ground	24	7	28
		Frozen	0	0	17
	Residue	Ground	4	0	0
3	Dropped	Ground	48	10	28
		Frozen	0	0	17
	Residue	Ground	0	0	0
4	Dropped	Ground	16	0	28
		Frozen	0	0	17
	Residue	Ground	2	0	0

Inoculum: 0·5 ml. of PR8 virus containing 1280 units of haemagglutinin.
Comparison of 'dropped' and residue areas, and of freezing or grinding of 'dropped' area.
The complement-fixing antigens were assayed with guinea-pig sera.
* Elementary body antigen.
† Soluble antigen.

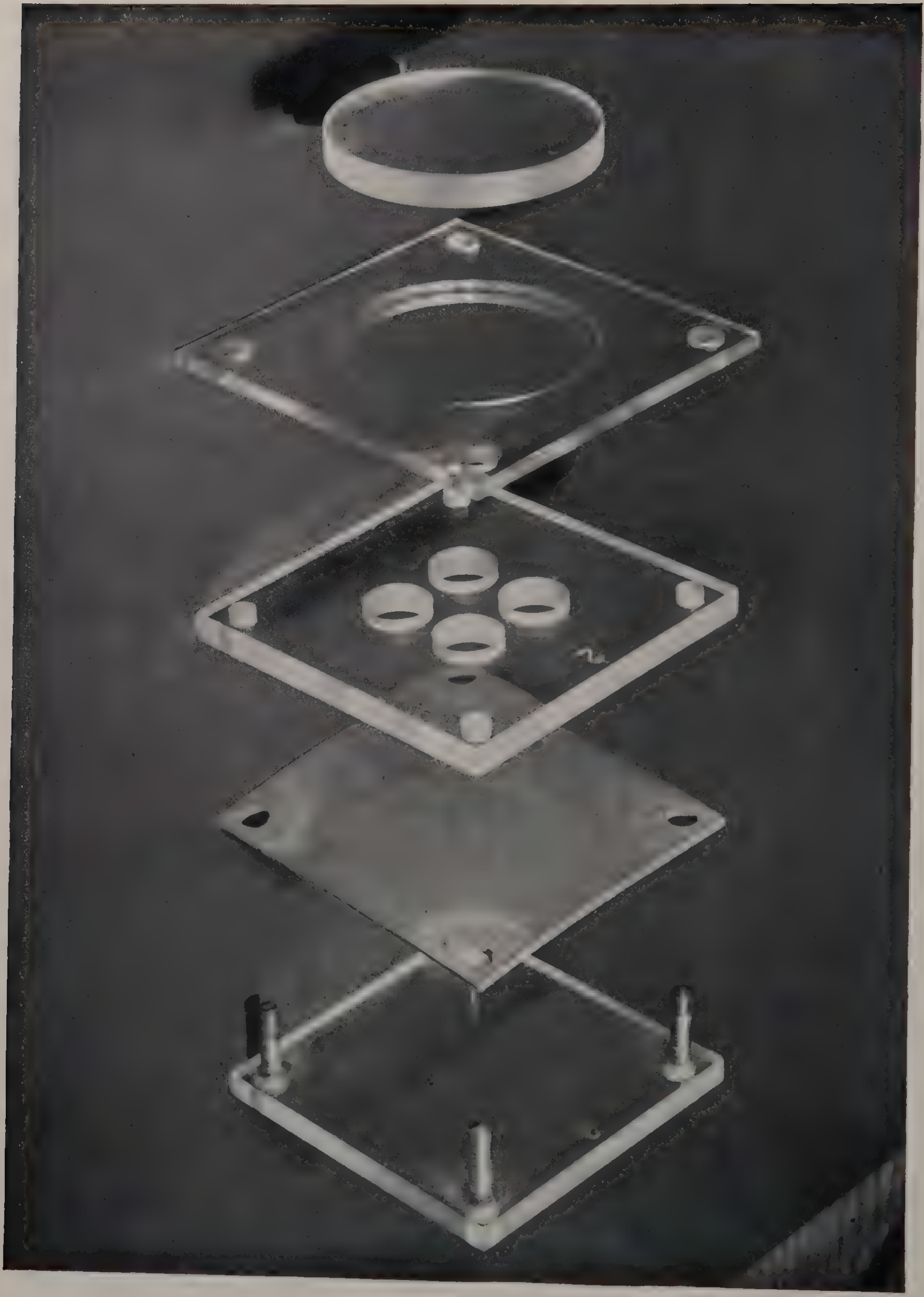
In this experiment the two techniques were applied to halves of the 'dropped' portion of the same membranes. It was essential to use the 'dropped' portion only for this comparison since multiplication of virus was largely confined to this part. Provided that the membranes are ground and treated with receptor-destroying enzyme, the assay of elementary bodies by the measurement of haemagglutinin is about as sensitive a method of detecting virus multiplication in the chorionic cells as assay of complement-fixing soluble antigen. The assay of elementary bodies by complement fixation was slightly less sensitive.

DISCUSSION

There are a number of ways in which multiplication of influenza virus expresses itself differently in the chorionic and the allantoic cells. Most strikingly, the chorionic cells seem to support a single cycle of multiplication only. The evidence for this is as follows. First, only a small rise in infectivity occurs after chorionic inoculation. Secondly, after a large inoculum the titre of soluble antigen rises rapidly and may be nearly maximal after 6 hr. After 48 hr. the titre has declined but this fall in titre can be prevented by re-inoculating with more virus. Thirdly, multiplication of virus is largely restricted to the area which is inoculated, that is, to the 'dropped' area. And finally, there is a



F. Fulton & A. Isaacs—Virus multiplication in the chick chorion. Plate 1



F. FULTON & A. ISAACS—VIRUS MULTIPLICATION IN THE CHICK CHORION. PLATE 2 proportion between the amount of virus inoculated and the yield of soluble antigen. In all these respects the chorionic cells behave differently from the allantoic but show a suggestive parallel to the mouse brain in its reaction to unadapted virus (Schlesinger, 1950; Cairns, 1951). The difference between the yield of elementary bodies from chorionic cells, depending on whether freezing and thawing or grinding is used, suggests that the elementary bodies are not readily released from the chorionic cells. As a working hypothesis, we visualize the elementary bodies in nests within the chorionic cells and surrounded by soluble antigen which is much more readily released by freezing and thawing.

REFERENCES

Burnet, F. M. & Stone, J. D. (1947). The receptor-destroying enzyme of *Vibrio cholerae*. *Aust. J. exp. Biol. med. Sci.* **25**, 227.

Cairns, H. J. F. (1951). The growth of influenza viruses and Newcastle disease virus in mouse brain. *Brit. J. exp. Path.* **32**, 110.

Fulton, F. (1949). Growth cycle of influenza virus. *Nature, Lond.* **164**, 189.

Fulton, F. (1952). The titration of influenza virus-neutralizing antibodies. *J. Hyg., Camb.* **50**, 265.

Fulton, F. & Armitage, P. (1951). Surviving tissue suspensions for influenza virus titration. *J. Hyg., Camb.* **49**, 247.

Fulton, F. & Dumbell, K. R. (1949). The serological comparison of strains of influenza virus. *J. gen. Microbiol.* **3**, 97.

Hoyle, L. (1948). Technique of the complement fixation test in influenza. *Mon. Bull. Minist. Hlth Lab. Serv.* **7**, 114.

Hoyle, L. (1950). The multiplication of influenza viruses in the fertile egg. *J. Hyg., Camb.* **48**, 277.

Hoyle, L. & Fairbrother, R. W. (1937). Antigenic structure of influenza viruses: the preparation of elementary body suspensions and the nature of the complement fixing antigen. *J. Hyg., Camb.* **37**, 512.

Isaacs, A. & Fulton, F. (1953). Growth of influenza virus in the chick chorion. *Nature, Lond.* **171**, 90.

Parker, R. C. (1938). *Methods of Tissue Culture*. New York: Hoeber.

Schlesinger, R. W. (1950). Incomplete growth cycle of influenza virus in mouse brain. *Proc. Soc. exp. Biol., N.Y.* **74**, 541.

EXPLANATION OF PLATES

Plate 1

A doublet: apparatus permitting infection of one side of the chorio-allantoic membrane or of both sides simultaneously.

Plate 2

A unit with four-hole face plate: apparatus permitting infection of only one side of the chorio-allantoic membrane.

(*Received* 16 *March* 1953)

ISAACS, A. & FULTON, F. (1953). *J. gen. Microbiol.* 9, 132–139.

Interference in the Chick Chorion

BY A. ISAACS
The National Institute for Medical Research, Mill Hill, London, N.W. 7
AND F. FULTON
The London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C. 1

SUMMARY: Experiments were carried out in an attempt to demonstrate in the chorionic cells of the chick chorio-allantoic membrane some of the interference phenomena with influenza viruses which are readily demonstrable in the allantoic cells. When a mixture of PR8 and Lee viruses was inoculated on the chorion, no interference occurred; on the contrary, the soluble antigen titres for A and B antigen showed a highly significant positive correlation in individual eggs. Influenza virus, partially inactivated by heat or ultraviolet irradiation and shown to produce 'auto-interference' in the allantoic cells, showed no such effect when inoculated on the chorion; on the contrary, a phenomenon which may be similar to that described under the name 'multiplicity reactivation' was observed. Influenza virus completely inactivated by heat or by ultraviolet irradiation caused pronounced interference with the multiplication of large inocula of virus in the allantoic cavity; no interference was found when the same materials were inoculated on the chorion. However, there is some evidence to show that irradiated virus caused slight inhibition of the increase in infectivity which results when small doses of influenza virus are inoculated on the chorion.

The results described in the preceding paper (Fulton & Isaacs, 1953) suggested that following inoculation of the PR8 strain of influenza virus on the chick chorion only a single cycle of virus multiplication occurred. When membranes were examined at different times after chorionic inoculation of virus it was found that the titres of soluble antigen were high at first, but had declined by 48–72 hr. and that this decline could be prevented by giving repeated chorionic inoculations of the same virus. In view of this finding it was interesting to see whether a state of refractoriness to influenza virus multiplication could be induced in the chorion by methods which have been found effective in the allantois.

MATERIALS AND METHODS

These have been described in the preceding paper (Fulton & Isaacs, 1953).

RESULTS

Effect of simultaneous chorionic injection of PR8 and Lee viruses

Within the allantoic cavity PR8 (influenza A) and Lee (influenza B) viruses are known to interfere with one another. Accordingly, eggs were injected on the chorion, with a single 0·5 ml. inoculum containing equal volumes of undiluted allantoic fluids infected with PR8 and Lee viruses. Controls were inoculated with 0·25 ml. of PR8 or Lee viruses mixed with 0·25 ml. of normal allantoic fluid. After 24 hr. incubation at 35° the membranes were harvested individually, frozen and thawed three times and titrated for soluble antigen with human convalescent sera from influenza A and B infections. The controls invariably showed a reaction with the homologous type serum only. In the eggs injected with both viruses simultaneously, positive reactions occurred with both sera. The titres, like those of the control eggs, showed a very wide scatter, but far from there being any interference, the titres for A and B antigen showed a highly significant positive correlation in individual eggs (see Fig. 1).

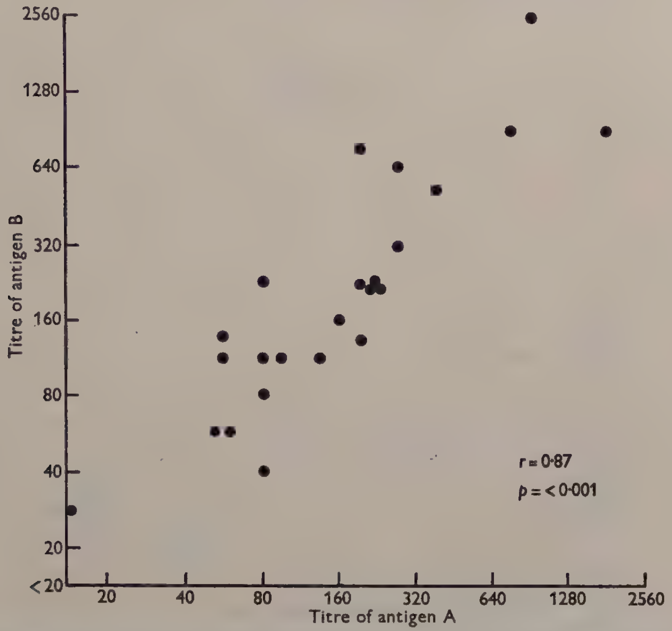


Fig. 1. Correlation of A and B soluble antigen titres in individual eggs injected on the chorion with a mixture of PR8 and Lee.

It appears, therefore, that some membranes produced high-titre and others low-titre soluble antigen, and that a single membrane reacted in a consistent manner to both viruses.

Effect of injecting eggs with partially inactivated virus

In eggs injected intra-allantoically with PR8 virus partially inactivated by heat or ultraviolet irradiation, an auto-interference phenomenon can be demonstrated (Henle & Henle, 1943). This is readily shown by injecting falling dilutions of the virus into eggs, harvesting after 3 days' incubation at 35° and titrating the allantoic fluids for haemagglutinin. The results of an experiment of this type with irradiated virus are shown in Fig. 2.

With virus irradiated for 10 sec. there is a clearly marked inhibition zone in the higher concentrations of virus due to an interference effect by the inactivated virus present. With less concentrated inocula the interfering virus is diluted beyond its effective level, and multiplication of the active virus in the preparation is unimpeded. With virus heated at 56° for 8 min., a slight inhibition zone was demonstrated in similar concentrations to those shown in Fig. 2 for irradiated virus; a clearer inhibition zone was demonstrable when larger inocula of heated PR 8 were used. This quantitative difference between virus inactivated by heat or ultraviolet light is in keeping with experiments by Henle & Henle (1944); we ourselves also find irradiated non-infective virus to be a more potent interfering agent than heated non-infective virus in experiments with influenza virus grown in the allantoic cavity.

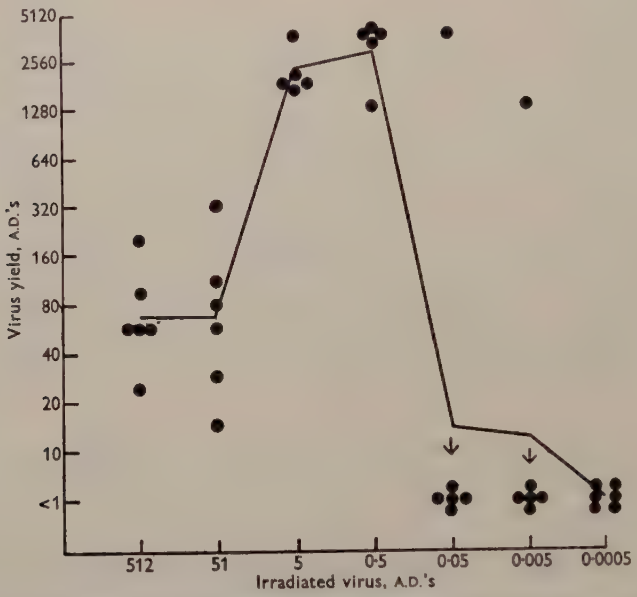


Fig. 2. Titres of agglutinin in the allantoic fluid following intra-allantoic inoculation of dilutions of partially inactivated (ultraviolet irradiation) PR 8 virus.

When these materials were inoculated on the chorion, no inhibition pro-zone was observed. On the contrary, a phenomenon which appears to resemble that described by Henle & Liu (1951) under the name 'multiplicity reactivation' was found. The results obtained with PR 8 virus heated at 56° for 8 min. are shown in Fig. 3, in which the titre of soluble antigen obtained in individual membranes is plotted as a function of the dose of virus inoculated.

Fig. 3 should be compared with Fig. 4 of Fulton & Isaacs (1953); the two experiments were carried out simultaneously. It will be seen that although the infectivity of the virus has dropped a thousandfold as a result of heating, the amount of soluble antigen produced by undiluted heated virus corresponded to about a tenfold dilution of the unheated PR 8. It appears, therefore, that considerably more soluble antigen was produced following chorionic inoculation of a large dose of heated PR 8 virus than would be expected on the basis of its egg infectivity titre.

In similar experiments with ultraviolet irradiated PR8 it was found that a proportion of eggs inoculated with undiluted virus produced more soluble and elementary body antigens than would be expected on the basis of the egg infectivity titre of the preparation. With further dilution no antigen production was detected. An experiment of this type is illustrated in Table 1.

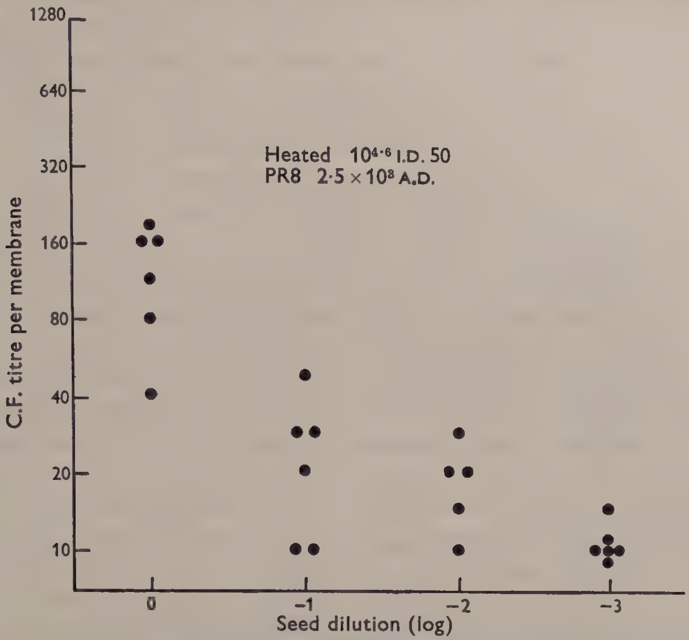


Fig. 3. Titres of soluble antigen in the chorio-allantoic membrane following inoculation on the chorion of dilutions of partially inactivated (heated) PR8 virus.

Table 1. *Effect of injecting a large and small dose of ultraviolet irradiated PR8 on the chorion*

		Complement-fixing titre	
Inoculum	Egg no.	Elementary body	Soluble antigen
Large dose 10^0 (5120 A.D. or 10^4 I.D. 50)	1	0	4
	2	0	0
	3	4	8
	4	0	4
	5	0	0
	6	4	8
Small dose 10^{-3} (5 A.D. or 10 I.D. 50)	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
	6	0	0

Complement-fixation tests were measured by the plate technique (see Fulton & Isaacs, 1953).

However, virus which was rendered wholly non-infective by heat or by ultraviolet irradiation did not yield soluble antigen or elementary bodies on inoculation on the chorion. These results are again similar to the 'multiplicity reactivation' phenomena described by Henle & Liu (1951). In addition, it is clear that no evidence of auto-interference was found when partially inactivated virus was inoculated on the chorion.

Effect of preliminary injection of inactivated virus on the multiplication of influenza virus

It is known from the work of the Henles and others that intra-allantoic inoculation of a large quantity of influenza virus inactivated by heat or by ultraviolet irradiation interferes with the multiplication of subsequently injected active virus. For the demonstration of maximal interference 24 hr. is an optimal interval between the two injections (Fazekas de St Groth, Isaacs & Edney, 1952). Accordingly, a number of eggs was injected on the chorion with a heavy inoculum of PR8 virus rendered non-infective by heating at 56° for 1 hr. The dose of virus used was 2560–3840 A.D., which was known from other experiments to cause pronounced inhibition (about 95 %) of the multiplication of PR8 virus in the allantoic cavity. This inoculation was followed 24 hr. later by a second injection of PR8 virus ($10^{7 \cdot 6}$ to 10^8 I.D. 50). After a further 24 hr. incubation the membranes were harvested individually and tested for the presence of soluble antigen and agglutinin. The titres were compared with those of control eggs injected at the same time with active virus and which had not received an initial injection. No evidence of interference was found in two experiments.

A similar experiment was carried out with PR8 virus inactivated by ultraviolet irradiation. 512 A.D. of irradiated virus was inoculated on to the chorion of six eggs, and at the same time six eggs were injected with similar material intra-allantoically. Controls were inoculated with broth-saline. All the eggs were re-inoculated 24 hr. later with 0·5 ml. undiluted PR8 allantoic fluid (containing 1280 A.D.) by the same route as the first injection and the allantoic fluids and membranes harvested after a further 24 hr. incubation. In this experiment soluble antigen and elementary body titres were measured by two techniques, and the results were essentially similar by both methods. The results by one technique are shown in Table 2.

It is clear from these results that there was considerable interference with soluble antigen and elementary body production in the eggs injected intra-allantoically with irradiated and active PR8. In the eggs similarly injected on the chorion, no interference occurred.

In the above experiments the assessment of interference was based entirely on titrations of soluble antigen and elementary bodies following inoculation of eggs with a large dose of active virus. It was of interest to see whether the small increase in infectivity following inoculation of small doses (10–100 I.D. 50) of PR8 virus on the chorion (Isaacs & Fulton, 1953) would be inhibited by a previous injection with inactive virus. Accordingly, groups of 10-day eggs were injected on the chorion with 512 agglutinating doses of irradiated and non-infective PR8 virus as used in the experiment shown in Table 2. Control eggs were injected with broth-saline. 24 hr. later the eggs were again injected on the chorion with 10 I.D. 50 of PR8 virus, and after a further 24 hr. the dropped portions of the membranes were harvested. They were ground, suspended in 1 ml. broth-saline, lightly spun and tested for infectivity by allantoic inoculation of 10-day eggs. Eleven eggs of each group were tested in three experiments, and the results are shown in Table 3.

Table 2. *Interference by irradiated virus with activity of PR8 virus given chorionically and intra-allantoically*

		Control eggs			Eggs injected with irradiated PR8		
Egg no.	Route of inoculation	Titre of HA fluid	SA total membrane	HA total membrane	Titre of HA fluid	SA total membrane	HA total membrane
1	Chorionic	<1	280	<40	<1	320	<40
2		<1	800	40	<1	560	<40
3		<1	240	<40	<1	280	<40
4		<1	320	<40	<1	480	40
5		<1	160	<40	·	·	·
1	Intra-allantoic	2560	>2560	3840	120	200	80
2		3200	>2560	2560	10	70	40
3		3840	2560	5120	40	120	40
4		3200	>2560	5120	40	60	40
5		3840	>2560	2560	240	320	80
6		2560	>2560	2560	120	140	80

Titre of HA fluid = titre of haemagglutinin per 0·25 ml. of allantoic fluid.
SA total membrane = soluble antigen in units per membrane using human serum.
HA total membrane = number of agglutinating doses per membrane after treatment with RDE.

Table 3. *Recovery of infective virus following chorionic inoculation of PR8*

Preliminary inoculation	Virus recovery (I.D. 50 per membrane)										
Broth-saline control	10^{1}	$10^{2\cdot8}$	$10^{4\cdot1}$	$10^{1\cdot5}$	0	0	$10^{2\cdot8}$	0	10^{1}	0	$10^{2\cdot5}$
Irradiated PR8	$10^{1\cdot3}$	0	0	$10^{2\cdot3}$	0	0	0	0	0	0	0

0 = <10 I.D. 50 recovered.

Table 3 shows the numbers of infective doses of virus recovered from individual membranes. In the control membranes considerable variation in the yield of infective virus was found in different eggs. This variation was greater than we had encountered earlier (Isaacs & Fulton, 1953), perhaps due to the smaller amount of virus inoculated or to the earlier time of harvesting the membranes in the present experiments. In eggs which had been previously injected with irradiated PR8 virus, there appears to be a distinctly lower yield of infective virus. The statistical significance of this result is not easily assessed as no information is available on the type of distribution of titres in the two groups. However, the number of eggs showing detectable virus in the control group (7/11) is significantly greater than in the interference group (2/11) on a simple probability basis ($p < 0{\cdot}05 > 0{\cdot}01$). This is the only evidence of an interference effect which we have observed in the chorionic cells.

DISCUSSION

The difficulty of demonstrating interference phenomena in chorionic cells, in contrast to the ease with which they could be shown in allantoic cells, suggested at first that there might be a greater number of sites of multiplication in the chorionic than the allantoic layer. The area of the 'dropped' portion of the chorionic layer is much less than half of that of the allantoic layer, but transverse sections of the chorio-allantoic membrane suggest that the chorionic cells may slightly outnumber the allantoic. However, this type of comparison is unprofitable since there is no means of knowing which cells in either layer support multiplication or at how many sites in a single cell multiplication of virus occurs. The slight interference effect of irradiated virus on the increase of infectivity following chorionic injection of small doses of virus suggests that the relative numbers of sites of multiplication in the chorionic and allantoic cells may not be important and focuses attention on other explanations.

One unusual finding in the present experiments may throw some light on this difficulty. From Table 2 it can be shown that in interference in the allantoic cells there is a disproportionately greater inhibition of production of elementary bodies than of soluble antigen. This effect is much greater in chorionic cells injected with a large amount of active virus, so that there is an extremely high ratio of soluble antigen to elementary bodies in eggs injected on the chorion as compared with eggs injected intra-allantoically. For example, from Table 2 in egg no. 2 of the control chorionic group the ratio of total elementary body to total soluble antigen units produced is 1/20, whereas in egg no. 3 of the control allantoic group the ratio is roughly 20/1 (assuming a volume of about 5 ml. of allantoic fluid). When small doses of virus are injected on the chorion an increase in the amount of elementary bodies can be inferred from the rise in infectivity which occurs. However, when large inocula (cf. Table 2) are used, the final yield of elementary bodies is smaller than was present in the original inoculum. That these elementary bodies are not simply remnants of the inoculum is suggested by the fact that they are not demonstrable in membranes until 4 hr. after inoculation. These facts suggest another explanation of the present results. When small amounts of virus are inoculated on the chorion the yield of elementary bodies is about 10–100 times the amount of virus inoculated, although with considerable egg variation. As the inoculum size increases, the yield increases more slowly. In support of this is the finding recorded by Fulton & Isaacs (1953) that a tenfold increase in the amount of virus inoculated corresponded to only a twofold increase in the yield of soluble antigen. Finally, with very large inocula, there is quite a low yield of elementary bodies. Presumably this inhibition is such that 'interference' produces no further reduction in yield.

We should like to thank J. E. Friend and E. Owen for their valuable technical help, and Miss M. V. Mussett who advised us on statistical evaluation of results.

REFERENCES

Fazekas de St Groth, S., Isaacs, A. & Edney, M. (1952). Multiplication of influenza virus under conditions of interference. *Nature, Lond.* **170**, 573.

Fulton, F. & Isaacs, A. (1953). Influenza virus multiplication in the chick chorio-allantoic membrane. *J. gen. Microbiol.* **9**, 119.

Henle, W. & Henle, G. (1943). Interference of inactive virus with the propagation of virus of influenza. *Science*, **98**, 87.

Henle, W. & Henle, G. (1944). Interference between inactive and active viruses of influenza. I. The incidental occurrence and artificial induction of the phenomenon. *Amer. J. med. Sci.* **207**, 705.

Henle, W. & Liu, O. (1951). Studies of host-virus interactions in the chick embryo-influenza virus system. VI. Evidence for multiplicity reactivation of inactivated virus. *J. exp. Med.* **94**, 305.

Isaacs, A. & Fulton, F. (1953). Growth of influenza virus in the chick chorion. *Nature, Lond.* **171**, 90.

(*Received* 16 *March* 1953)

Gompels, A. E. H. (1935). *J. gen. Microbiol.* 9, 140–148.

Antigenic Relationships of Swine Influenza Virus

By ANNETTE E. H. GOMPELS

National Institute for Medical Research, Mill Hill, London, N.W. 7

SUMMARY: Antigenic comparisons of six strains of swine influenza virus with three strains of human influenza A virus were made by the agglutination-inhibition technique. The strains were also compared in regard to inhibition by normal sera, rate of elution from red cells, pathogenicity for mice, and other properties. All swine strains were significantly different from human strains. Four American swine strains formed a fairly homogeneous group, but were less closely related to two strains recovered in the United Kingdom. The findings are briefly discussed in terms of the relationship between human and swine influenza.

Human influenza A viruses recovered from succeeding epidemics exhibit varying degrees of difference in antigenic structure. In order to ascertain whether this occurred among strains of swine influenza virus, the antigenic structure of standard strains of swine influenza virus was compared with that of some more recently isolated strains.

The earliest work on this subject was carried out by Shope (1935–6, 1936, 1937*a*, *b*, 1938) in his extensive researches on swine influenza. Following studies on immunological and serological relationships between swine and human viruses, Shope concluded in agreement with Laidlaw (1935) that swine influenza might well have had its origin from the 1918–19 human influenza pandemic virus.

Isolation of strains of swine influenza in the British Isles (Lamont, 1938; Blakemore & Gledhill, 1941) was followed by a survey of the antigenic relationship of British swine influenza strains to standard human and swine influenza viruses by means of the agglutination-inhibition tests with chicken and ferret antisera by Hudson, Sigel & Markham (1943) and by Glover & Andrewes (1943). Since then, however, methods have been evolved for the destruction of non-specific inhibitor in normal sera, and serological relationships, as indicated by haemagglutination-inhibition tests, can now be more clearly demonstrated. It is interesting to review the relationship between human and swine influenza viruses in the light of recent techniques.

MATERIALS

Virus strains. The standard laboratory strains of swine influenza, Shope 15 (1931) and Cambridge (Blakemore & Gledhill, 1941) and the standard strains of human influenza A were obtained from the World Influenza Centre (W.I.C.). One Northern Ireland Standard strain (Lamont, 1938) and two American strains of swine influenza, Felin 1949 and Vogt 1946, were obtained through the courtesy of Dr R. E. Shope. A third American strain of swine influenza virus, Wisconsin 1949, was obtained through Prof. W. I. B. Beveridge from Dr Kissling.

All strains were passed allantoically in 10-day-old chick embryos. After

48 hr. incubation at 35° the allantoic fluids were chilled, harvested, lightly centrifuged and the supernatants titrated and stored in capillary tubes and ampoules at $-70°$. Subsequent egg passes for allantoic fluid material were made from this stock.

Sera. Normal ferret serum and standard antisera from the W.I.C. were used for serological tests.

Ferret antisera were prepared against Wisconsin, Felin, Cambridge and Northern Ireland strains by bleeding ferrets 12 days after intranasal instillation of 1 ml. of freshly harvested allantoic fluid diluted 10^{-2} in broth saline.

For haemagglutination-inhibition tests all sera were treated with R.D.E. eluate (Van der Veen & Mulder, 1950; Isaacs & Bozzo, 1951).

Receptor-destroying enzyme (R.D.E.). An extract of the 4Z strain of *Vibrio cholerae* containing the R.D.E. was prepared by the method of Burnet & Stone (1947), and from this an eluate was prepared by absorption and elution on fowl red cells. The preparation of R.D.E. used in these experiments had a titre of 1600 units/ml. as tested with MEL virus (Burnet & Stone, 1947).

Red cells. A standardized ½ % chick erythrocyte suspension was used for all titrations except where specified.

R.D.E.-treated red cells. Dilutions of R.D.E. were prepared in calcium-acetate buffer, pH 6·0. To 4 ml. volumes of each dilution, 0·1 ml. of packed fowl red cells was added and incubated for ½ hr. at 37°. Sodium citrate was then added to give a final concentration of 2 % and the cells were centrifuged, washed in 2 % citrate saline and finally suspended in 2 % citrate saline to make a 1 % red cell suspension.

METHODS

Complement-fixation tests were carried out using a modification of Hoyle's (1948) technique. Soluble antigen was prepared from infected chorio-allantoic membranes and titrated against convalescent A and B human influenza antisera.

Haemagglutination-inhibition tests. Serial twofold dilutions (0·25 ml.) of serum were prepared in normal saline and an equal volume of ½ % fowl red cells was added to each dilution, followed by eight agglutinating doses (8 A.D.) of virus in 0·25 ml. The cells were allowed to settle at room temperature, and readings were made by the pattern method. Partial, one plus (+) agglutination-inhibition was taken as the end-point. 1 A.D. of virus is defined as the least amount of virus which will produce partial or one plus agglutination.

Treatment of sera. To reduce the non-specific inhibitor present in normal sera, ferret sera were incubated overnight at 37° with a suitable dilution of R.D.E. and then inactivated by heating at 56° for 1 hr.

Test of destruction of non-specific serum inhibitor. In a preliminary test, the amount of non-specific inhibitor present in R.D.E. treated and untreated sera was estimated by titration of the so-called Francis inhibitor, i.e. measuring the inhibitory titre of serum for heated influenza B virus.

Serial twofold dilutions (0·25 ml.) of antiserum in normal saline were prepared, and an equal volume of 5 A.D. of influenza B virus previously heated for ½ hr. at 56°, was added to each dilution. This was left to stand at room temperature for ½ hr. before adding a final volume of 0·25 ml. of ½ % inhibitor-sensitive fowl cells (Anderson, Burnet & Stone, 1946). The cells were left to settle at room temperature, and readings were made in the same way as in the anti-haemagglutinin titration. Reduction to 1 % of the amount of non-specific inhibitor present in the untreated ferret serum was considered sufficient, since the amount remaining would not interfere with the readings of the haemagglutination-inhibition titrations.

Absorption and elution from red cells. Experiments on the comparative rates of elution of swine influenza virus strains from fowl red cells were carried out on 1 % suspensions of cells in virus diluted to a concentration of 320 A.D. per 0·25 ml. Absorption was carried out at 2° for ½ hr., and elution at 37° over a period of 24 hr.

Fowl red cell receptor gradient. The relative positions of the swine influenza strains on the receptor gradient were assessed with R.D.E.-treated chick cells using the method adopted by Burnet, McCrea & Stone (1946).

Mouse and egg infectivity tests were carried out using the same inocula for albino mice, and 10-day egg embryos. Each mouse received 0·05 ml. of inoculum intranasally under light anaesthesia, and deaths were recorded over a period of 14 days. Thereafter, the lung lesions of the survivors were examined. Each egg was inoculated allantoically with 0·05 ml. of inoculum and harvested after 3 days' incubation at 35°. The dilutions at which 50 % of the eggs were infected, E.I.D. 50, and at which the mice showed 50 % lung lesions, were determined according to the method described by Reed & Muench (1938).

Cross-neutralization tests were carried out on 10-day chick embryos. Equal volumes of suitably diluted virus and dilutions of inactivated serum were mixed and allowed to stand for 15 min. at 2°. Eggs were inoculated allantoically with 0·05 ml. serum virus mixture and incubated at 35° for 3 days.

RESULTS

Complement-fixation tests on the strains of swine and human influenza demonstrated the existence of a common soluble antigen for human A influenza and swine influenza, but no relationship between human B influenza and swine influenza viruses (see Table 1). No quantitative differences were observed between the titres with human serum titrated against human or swine influenza.

Haemagglutination-inhibition tests were carried out to determine the extent to which swine influenza strains would be inhibited by normal ferret serum. Of the six strains of swine influenza, the Cambridge and Northern Ireland strains were significantly inhibited by normal ferret serum (see Table 2). No strains of human influenza A or B viruses are known which are not inhibited to a significant titre by normal ferret serum.

Subsequent experiments on the nature of this non-specific inhibitor proved it to be stable after heating for 30 min. at 65° but sensitive to treatment with R.D.E. eluate. Similar properties have been noted by Isaacs & Bozzo (1951) for the non-specific inhibitor of human influenza strains in normal ferret serum. The inhibitor has therefore similar properties to the 'Francis' inhibitor.

Table 1. *Complement-fixation test*

Titration of influenza soluble antigens against human influenza A and B convalescent sera

Soluble antigen	Antigen titre with		
	1/16 A serum	1/16 B serum	Buffer control
Swine:			
Shope	8	<2	<2
Cambridge	16	<2	<2
Vogt	8	<2	<2
Felin	7	<2	<2
Wisconsin	20	<2	<2
Northern Ireland	32	<2	<2
Human:			
A	96	<2	<2
B	<2	32	<2

Table 2. *Inhibition of swine and standard human strains of influenza by pooled normal ferret serum*

Virus strains	Haemagglutination-inhibition titre
Swine:	
Shope 15	<10
Cambridge	200
Vogt	<10
Felin	<10
Wisconsin	<10
Northern Ireland	15
Human:	
PR8	40
WS–38	15
FM1	100

Table 3. *Titres of ferret sera against human and swine influenza viruses*

Virus	Year of isolation	Serum							
		Shope	Northern Ireland	Cambridge	Wisconsin	Felin	WS	PR8	FM1
Shope	1931	960	<10	160	20	80	<10	<10	<10
Northern Ireland	1938	<10	2560	<10	—	—	320	<10	<10
Cambridge	1941	3840	60	5120	20	20	200	<10	100
Wisconsin	1949	100	<10	<10	240	320	<10	<10	<10
Vogt	1946	160	<10	<10	80	800	<10	<10	<10
Felin	1949	160	<10	<10	160	1280	<10	<10	<10
WS	1933	<10	<10	<10	<10	40	5120	40	<10
PR8	1934	<10	<10	<10	<10	20	160	5120	<10
FM1	1947	<10	<10	<10	<10	<10	<10	<10	1280

The results of the haemagglutination-inhibition tests using R.D.E.-treated ferret sera prepared against swine and influenza viruses (Table 3) showed close, though not identical, antigenic relationships among the American swine influenza strains. However, no such relationships were found between the swine influenza strains and any of the main subtypes of the human influenza A strains.

It is interesting to note that the Shope strain (1931) shows a fairly close antigenic relationship with the more recently isolated strains of Vogt, Wisconsin and Felin which is in contrast to the divergence of antigenic structure exhibited in comparable tests with earlier (WS and PR8) and more recently isolated (FM1, 1947) strains of human influenza A virus.

The Northern Ireland strain showed no antigenic relationship to the American swine strains, and was apparently only distantly related to the Cambridge swine strain. Both the Northern Ireland and the Cambridge strains were inhibited by WS antiserum, and the latter also by FM1 antiserum. The Cambridge strain, however, exhibited a significant antigenic relationship with the Shope strain, although not with Vogt, Felin and Wisconsin, American strains related to Shope 15.

WS and PR8 viruses were inhibited to low titre by Felin serum. This was the only swine antiserum which showed inhibition of the human influenza virus strains.

Cross-neutralization tests. In an attempt to confirm the relationships between Cambridge swine and human influenza A viruses found in the haemagglutination-inhibition tests, cross-neutralization tests were carried out in eggs.

Table 4 shows comparative titres of a number of sera tested in haemagglutination-inhibition tests and egg neutralization tests. Although no direct correspondence was found between the titres in the different tests, there is a general similarity in the results of the two tests, except for the results with Felin serum.

Table 4. *Relationship between egg neutralization and haemagglutination-inhibition tests with Cambridge virus*

Antiserum	50 % virus neutralization titre in eggs	50 % haemagglutination-inhibition titre against 8 A.D. virus
Swine:		
Shope 15	70	3840
Cambridge	>320	5120
Felin	40	20
Human:		
FM1	4	100
WS	6	200
PR8	<4	<10
Normal ferret	<4	<10

Rates of elution of virus from red cells. Comparative studies made on the rates of elution of 320 A.D. per 0·25 ml. of each strain of swine influenza virus from a 1 % suspension of chicken erythrocytes showed complete elution of the Cambridge strain within an hour, while Shope 15 strain failed to elute completely even after 24 hr. incubation. The remaining strains range between these two extremes—see Fig. 1.

Red cell receptor gradient. These results prompted an investigation into the comparative positions of individual swine influenza strains on the red cell receptor gradient. Table 5 shows the titre of R.D.E. which removed red cell receptors to a standard degree, i.e. to produce 50 % agglutination by 5 A.D. of the test virus.

Experiments with R.D.E.-treated erythrocytes showed a fairly uniform behaviour of the swine strains with the exception of Cambridge virus which was a little 'earlier' in the gradient.

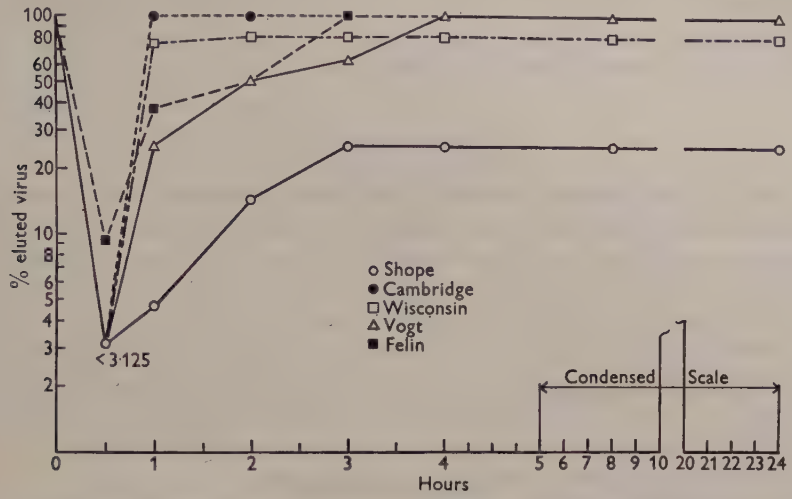


Fig. 1. Comparative rates of elution of swine influenza virus strains from fowl red cells.

Table 5. *Agglutination of* R.D.E.*-treated fowl red cells by* 5 A.D. *of swine influenza virus strains*

Virus strain	Dilution of R.D.E. at which 50 % agglutination occurred
Shope 15	80
Vogt	80
Felin	120
Wisconsin	80
Cambridge	240
Northern Ireland	80

Table 6. *Comparison of egg infectivity and mouse pathogenicity of the swine influenza strains*

Titres show infectivity per 0·05 ml. allantoic fluid

Virus	A. Egg infectivity, E.I.D. 50	B. Mouse pathogenicity, 50 % lung lesion dose	Ratio A:B (in logs)
Cambridge	$10^{-7{\cdot}5}$	$10^{-5{\cdot}5}$	2
Northern Ireland	$10^{-7{\cdot}5}$	$10^{-6{\cdot}7}$	0·8
Shope 15	$10^{-7{\cdot}7}$	$10^{-7{\cdot}5}$	0·2
Vogt	10^{-6}	$10^{-2{\cdot}5}$	3·5
Felin	$10^{-6{\cdot}5}$	$10^{-2{\cdot}5}$	4
Wisconsin	10^{-7}	10^{-2}	5

Egg and mouse infectivity tests. It has been shown that the previous passage history of a virus can play an important part in altering its pathogenicity and infectivity titres in any particular host.

Table 6 is a comparison of concurrent egg and mouse infectivity tests carried out on the stock material used in these experiments. These tests showed that the swine influenza viruses varied considerably in their mouse pathogenicity. While the adapted Standard Laboratory strains, e.g. Shope 15, Cambridge and Northern Ireland showed high titres in mice, the more recently isolated, unadapted strains, e.g. Vogt and Felin, showed in contrast comparatively low mouse infectivity titres.

DISCUSSION

The relationships between human A and swine influenza have been an intriguing problem for some time. Apart from the fact that the two clinical diseases are not dissimilar, the causative viruses show a number of striking similarities.

(1) Both viruses will grow in swine, ferrets and mice after intranasal instillation.

(2) Both viruses will grow in the amniotic and allantoic cavities of chick embryos.

(3) Both viruses agglutinate fowl and mammalian blood cells.

(4) The viruses share a common soluble antigen.

There are three possibilities which might explain these relationships.

The first possibility is that the viruses of swine and human influenza are closely related, with swine influenza acting as a reservoir for human influenza. In support of this is the occurrence of influenza A in pigs after experimental infection with human influenza A viruses (Shope & Francis, 1936) and as a result of swine becoming naturally infected with human influenza virus (Shope, 1938). However, no instances have been recorded of the reverse procedure, i.e. man becoming infected with swine influenza either experimentally or as a result of coming in contact with swine suffering from the disease. Again, cross-haemagglutination-inhibition tests with immune ferret sera demonstrate that there is no close antigenic relationship between recently isolated swine and recently isolated human influenza viruses.

This leads to the consideration of a second possibility suggested by Laidlaw (1935) and subscribed to by Shope (1935–6) that the swine influenza virus might represent a surviving prototype of the human virus of the 1919 pandemic. In that case it would appear that the human, but not the swine viruses, have diverged widely in antigenic relationship from the originally isolated strains. The swine strains of Shope 15 and Felin isolated 18 years apart, show remarkable similarities in their antigenic relationships, lack of inhibition by normal ferret serum, and position in the red cell receptor gradient. It is interesting to note, however, that there are some biological differences, e.g. pathogenicity in mice, between Shope 15 and more recently isolated swine strains. On the other hand, striking serological differences have been found in human A strains isolated between 1933 and 1947 (Van der Veen & Mulder, 1950). These facts, taken along with the evidence from studies of human sera, are against any hypothesis that human influenza A viruses are at present evolving from swine influenza viruses.

A third possibility is that human and swine influenza viruses may have arisen from a common prototype in the past, and that human and swine strains, while retaining a few common characteristics, have since evolved along rather different antigenic lines.

It is possible that swine influenza may have become endemic in the United States, and that the original Shope 15 strain isolated in 1931 has largely maintained its biological characters since then. This is shown by its close serological relationships with the more recently isolated strains of Vogt 1946 and Felin 1949. It is, however, doubtful whether swine influenza is still prevalent in Europe. There have been no reports of it since 1941 (Blakemore & Gledhill), and although there is evidence of a widespread chronic virus pneumonia of pigs (Beveridge & Gulrajani, 1951; Betts, 1952), the aetiological agent is apparently quite unrelated to that of swine influenza.

Much gratitude is due to Dr C. H. Andrewes, F.R.S., and Dr A. Isaacs for constant help and advice.

This work was carried out while the author was in receipt of a personal grant from the South African Institute for Medical Research, Johannesburg.

REFERENCES

Anderson, S. G., Burnet, F. M. & Stone, J. D. (1946). Modified Salk test for *in vitro* titration of influenza antibodies. *Aust. J. exp. Biol. med. Sci.* **24**, 269.

Betts, A. O. (1952). Respiratory diseases of pigs. V. Some clinical and epidemiological aspects of virus pneumonia of pigs. *Vet. Rec.* **64**, 283.

Beveridge, W. I. B. & Gulrajani, I. S. (1951). Studies on respiratory diseases of pigs. IV. Transmission of infectious pneumonia and its differentiation from swine influenza. *J. comp. Path.* **61**, 118.

Blakemore, F. & Gledhill, A. W. (1941). Swine influenza in the British Isles. *Proc. R. Soc. Med.* **34**, 611.

Burnet, F. M., McCrea, J. F. & Stone, J. D. (1946). Modification of human red cells by virus action. I. The receptor gradient for virus action in human red cells. *Brit. J. exp. Path.* **27**, 228.

Burnet, F. M. & Stone, J. D. (1947). The receptor destroying enzyme of *V. cholerae*. *Aust. J. exp. Biol. med. Sci.* **25**, 227.

Glover, R. E. & Andrewes, C. H. (1943). The antigenic structure of British strains of swine influenza virus. *J. comp. Path. Therap.* **53**, 329.

Hoyle, L. (1948). Technique of the complement-fixation test in influenza. *Mon. Bull. Min. Hlth*, p. 114.

Hudson, N. P., Sigel, M. M. & Markham, F. S. (1943). Antigenic relationship of British swine influenza strains to standard human and swine influenza viruses. *J. exp. Med.* **77**, 467.

Isaacs, A. & Bozzo, A. (1951). The use of *V. cholerae* filtrates in the destruction of non-specific inhibitor in ferret serum. *Brit. J. exp. Path.* **32**, 325.

Laidlaw, P. P. (1935). Epidemic influenza: a virus disease. *Lancet*, i, 1118.

Lamont, H. G. (1938). The problems of the practitioner in connection with the differential diagnosis and treatment of diseases of young pigs. *Vet. Rec.* **50**, 1377.

Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *Amer. J. Hyg.* **27**, 493.

Shope, R. E. (1935–6). The influenzas of swine and man. *Harvey Lect.* p. 183.

Shope, R. E. (1936). Immunization experiments with swine influenza virus. *J. exp. Med.* **64**, 47.

Shope, R. E. & Francis, T. F., Jr. (1936). The susceptibility of swine to the virus of human influenza. *J. exp. Med.* **64**, 791.

Shope, R. E. (1937*a*). Recent knowledge concerning influenza. *Ann. intern. Med.* **11**, 1.

Shope, R. E. (1937*b*). Immunological relationship between the swine and human influenza viruses in swine. *J. exp. Med.* **66**, 151.

Shope, R. E. (1938). Serological evidence for the occurrence of infection with human influenza virus in swine. *J. exp. Med.* **67**, 739.

Van der Veen, J. & Mulder, J. (1950). *Studies of the Antigenic Composition of human Influenza Virus A Strains.* Leiden: Stenfert Kroese.

(*Received* 20 *March* 1953)

Wells, A. Q. (1953). *J. gen. Microbiol.* **9**, 149.

Mycobacterium tuberculosis var. *muris*

By A. Q. WELLS
Sir William Dunn School of Pathology, University of Oxford

SUMMARY: The valid name for the acid-fast bacillus which causes tuberculosis in voles is *Mycobacterium tuberculosis* var. *muris* Brooke.

The object of this paper is to discuss briefly the name for the mycobacterium which causes tuberculosis in voles (Wells, 1937), a full description of which has already been published (Wells, 1946). The mycobacterium has been called the vole acid-fast bacillus (Wells & Brooke, 1940); the murine type of tubercle bacillus (Wells, 1946); *Mycobacterium muris* (*Topley & Wilson's Principles*, 1946) and *Myco. tuberculosis* var. *muris* (Brooke, 1941). The name *Myco. muris* has already been given to a mycobacterium isolated by Simmons (1927) from the intestinal contents of a house mouse. His description of the cultural requirements and pathogenicity of this bacterium clearly shows that it is quite different from the mycobacterium isolated from voles. The name *Myco. muris* is not therefore available for the latter.

It is not necessary to recapitulate the details of the experimental work already published (Wells, 1946), which lead to the conclusion that the bacterium causing tuberculosis in voles is a new type of tubercle bacillus. If this work is accepted, the proper name for the mycobacterium would seem to be *Mycobacterium tuberculosis* var. *muris* Brooke.

REFERENCES

Brooke, W. S. (1941). The vole acid-fast bacillus. *Amer. Rev. Tuberc.* **43**, 806.
Simmons, J. S. (1927). An acid-fast organism isolated from a mouse. *J. infect. Dis.* **41**, 13.
Topley and Wilson's Principles of Bacteriology and Immunity (1946). 3rd ed. revised by Wilson, G. S. & Miles, A. A. London: Arnold.
Wells, A. Q. (1937). Tuberculosis in wild voles. *Lancet*, i, 1221.
Wells, A. Q. & Brooke, W. S. (1940). The effect of vaccination of guinea-pigs with the vole acid-fast bacillus on subsequent tuberculous infection. *Brit. J. exp. Path.* **21**, 104.
Wells, A. Q. (1946). The murine type of tubercle bacillus (The vole acid-fast bacillus). *Spec. Rep. Ser. med. Res. Coun., Lond.* no. 259.

(*Received* 20 *March* 1953)

No. 1, Volume 9 of the Journal of General Microbiology was issued on 26 August 1953.

WOODS, D. D. (1953). *J. gen. Microbiol.* 9, 151–173.

The Integration of Research on the Nutrition and Metabolism of Micro-organisms

The Inaugural Marjory Stephenson Memorial Lecture

BY D. D. WOODS
Microbiology Unit, Department of Biochemistry, University of Oxford

---

CORRIGENDUM

In ANDERSON, E. S. & FELIX, A. (1953), *J. gen. Microbiol.* 9, 65–88.

On p. 73, line 29, 10^{-6} *should be* 10^{-9}.

Stephenson summarized her own experience and thought and drew for us a simple overall picture of the various methods of approach to research in microbiology, and especially chemical microbiology. I have always felt this to be one of those clear-sighted and simple statements of general principles which remain indefinitely helpful and become, so to speak, part of the common-law of a science. Unfortunately, we have no published account of this actually from her own pen, but there is a summary in a report of the meeting (Anonymous, 1945: see also Elsden & Pirie, 1949; Woods, 1950*a*). She defined five levels at which research was carried out both from the biological and biochemical aspects. It was particularly emphasized that no one level was more important than another: they represent a spectrum rather than a ladder. Let us examine these levels (Fig. 1) briefly from the point of view of chemical microbiology in particular.

Perhaps the ultimate problem is to explain in biochemical terms the life of microbes in the mixed associations in which they usually exist in nature. It is unlikely that we shall dig very deep at this level until we know more about the precise chemical nature of the environment, the number and nature of the organisms present, and their biochemical potentialities: all these factors are likely to be changing constantly.

The first stage of simplification is, of course, restriction of the microbial population to a single species. The environment remains complex and undefined chemically. Here we hope to find out, from a comparison of the initial and spent medium, something of the overall metabolic activities of the microbe. It is of course at this level too that the great bulk of biological investigation takes place.

Mixed cultures: complex 'natural' media [Organisms in natural environment]	Growing cultures
Pure cultures: complex undefined media [General microbiology: overall metabolic picture]	
Pure cultures: chemically defined media [Nutrition: biochemical mutants]	
Cell suspensions (pure cultures): defined substrates [Overall products: intermediary metabolism]	Viable: usually no growth
Cell preparations: defined substrates (*a*) 'Complete'—mixtures of enzyme systems (*b*) 'Fractions'—multienzyme systems (*c*) 'Purified'—isolated enzymes [Further detail of individual reactions]	Non-viable

Fig. 1. Levels of investigation in chemical microbiology.

At the next level the environment is simplified. We endeavour to establish in chemical terms the minimum requirements for optimum growth. This is the branch of our work usually called 'nutrition'.

Further simplification of the environment in order to study the action of a microbe on a single or relatively few substrates usually (though not always) results in conditions in which growth cannot occur. In order to obtain sufficient material, and to define the conditions more rigidly, we normally employ washed suspensions of cells harvested from a suitable growth medium. At this level we find out the overall products of action on defined substrates and, by traditional biochemical techniques, hope to begin breaking down metabolic processes into their individual steps.

The detailed analysis of a metabolic pathway must finally require the study of individual reactions as catalysed by individual enzyme systems. This is likely to yield most information if the enzyme can be extracted from the cell and purified. Furthermore, certain substances, which may be formed as intermediates within the cell and further metabolized therein, do not gain access to the cell when supplied externally; they can only be studied (if the enzymes survive) by non-viable cell preparations in which permeability barriers have been destroyed.

There is, of course, no clear-cut separation of these different levels; one can always see intermediate stages. At one end of the spectrum we are in closest relation to purely biological matters, and it is here that many of our problems are set. At the other end we become almost pure biochemists, and it is here that we must not lose sight of our main objectives and regard micro-organisms simply as a convenient biochemical material.

Marjory Stephenson emphasized the importance of tackling a given problem at as many of these levels as possible. Research at one level may frequently come to a stop until new facts and new ideas become available from research at a different level. Put in a different way, these methods of approach represent the *tactics* of our attack upon a problem. The grand *strategy* is a co-ordinated attack on several fronts, shifting the main weight from one front to another as the results dictate and according to the availability of our experimental armament.

Apart from experimental convenience there are equally cogent reasons to subject results obtained at one level to rigorous test at others. To take one example only, it is clear that the work on adaptation and adaptative enzyme formation emphasizes the ability of the cell or culture to change both qualitatively and quantitatively its overall enzymic constitution.* The activities of washed suspensions of cells harvested at one particular stage of growth (usually towards the end in order to obtain enough cells) give us only a cross-section of the biochemical potentiality at that time. What we need eventually is an integration of all the metabolic events since the time of inoculation. This is provided by work with growing cultures but is more difficult to analyse; we must at least therefore try to discover, by reference to work at this level, whether the reactions we find with cell suspensions are indeed important for the growth of a culture. The adaptive powers of micro-organisms, though providing us with a valuable weapon in our experimental armament, do also complicate matters by adding, so to speak, a fourth dimension to our system of microbe, substrates and time.

For the remainder of this lecture I want to try to illustrate in particular how the integration of work at the several different levels covering nutrition and metabolism has advanced our knowledge of the synthetic mechanisms of the cell. Since co-ordinated biosynthesis is at its optimum in a growing culture it is natural to hope that work with such systems may provide valuable clues.

In attempting to give a broad outline, it is inevitable that I shall piece together so many individual contributions that it will be impossible to give proper acknowledgment to all those whose work is implicated. I am sure that for the purpose of doing honour to Marjory Stephenson those concerned will not mind, if, without permission, I nominate myself as their spokesman. Time will not permit me to recapitulate how these ideas grew up and proved their value. Also, since most members of the Society are not professional biochemists, I will try to set them out first in non-chemical terms, and then illustrate them with, I fear, woefully few concrete examples of their application.

---

* See, for example, the contributions to the Symposium on Adaptation in Micro-organisms (various authors, 1953) held at this meeting of the Society.

Pathways of biosynthesis

Let us consider a number of pathways leading to substances such as C, D and Z (Fig. 2) which are known essential metabolites necessary for the formation of new cell material. The information which we seek is (*a*) the chemical nature of the primary intermediates such as A, B, X, Y and so on, and (*b*) the properties of the enzyme systems catalysing individual steps such as B→C and X→Y. The available methods of approach through studies of nutrition all depend really on finding what substances are needed to restore growth when there is some biochemical lesion, either natural or induced, which results in a failure at one step of a sequence of reactions.

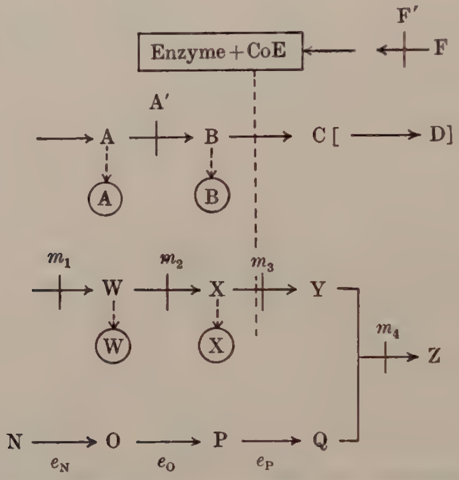


Fig. 2. Scheme illustrating some different methods of experimental approach used in studies of biosynthesis. The symbols are explained in the text.

Types of metabolic lesion

Requirement for growth factors. The very fact that a number of organisms are unable to grow unless certain essential growth factors are provided is in itself a proof of failure to synthesize these substances and of the existence of a metabolic lesion compared with competent organisms. Such growth factors are of two main types: (*a*) substances incorporated into main cell constituents, e.g. amino-acids, and (*b*) substances of ultimate catalytic function such as members of the vitamin B group. The function of the latter, whenever it has been determined, has proved to be as part of the structure of coenzymes or prosthetic groups of important enzyme systems of the cell. This possibility may now be adopted as a working hypothesis in the case of new growth factors of unknown function.

Apart from organisms normally requiring growth factors, it is possible to obtain mutants (by irradiation, treatment with certain chemicals, etc.) which have lost the ability to synthesize particular essential metabolites. The failure may be due in individual cases (including organisms with natural growth-factor requirements) to (*a*) the absence of an enzyme protein, (*b*) an enzyme with altered properties functional only under changed growth conditions, (*c*) the presence of an inhibitory substance, or (*d*) a failure to produce a coenzyme or prosthetic group.

Induction by substances similar in chemical structure to essential metabolites. Substances of similar chemical structure to a growth factor or intermediate (e.g. A^1 to A, F^1 to F in Fig. 2) often prevent the utilization of a metabolite by combining with and competing for the enzyme concerned. This in effect induces a lesion of the same type as that already discussed. Inhibition is overcome ideally (*a*) competitively by the metabolite itself, and (*b*) by the product of the inhibited reaction irrespective of the concentration of the inhibitor.

Failure to produce a coenzyme. If the growth factor is of the catalytic type (F), its absence will result in a failure to produce the coenzyme form (CoE, Fig. 2), and thus again a failure to carry out a particular enzyme reaction. Restriction of F may be attained either by omitting it from the culture medium, or by adding a chemical analogue of F which specifically inhibits its utilization. Growth may be restored either by adding the coenzyme itself or an intermediate in its formation (i.e. substances of the same chemical type as F), or by adding the product (C) of the inhibited reaction (i.e a substance probably of dissimilar chemical type to F).

If the coenzyme is required, as is often the case, for two or more enzyme systems, then all the products (i.e. C and Z) will have to be added to restore growth. Addition of C alone would be expected only to reduce the amount of F required, but not to abolish the requirement.

Nature of intermediates

'*Replacement*' *experiments.* Suppose that we have a series of organisms in which metabolic lesions of this general type are present, or have been induced, with the result that all the organisms require the same factor (say Z) for growth. It is unlikely that all will have failed at precisely the same step in the synthesis of Z; some may fail to convert W to X, others X to Y and so on, i.e. the lesions are all within one synthetic pathway but at different points. Then with some organisms growth would be restored by Z only; with others by Y or Z but not W or X; with still others by X, Y or Z but not W. If, therefore, we test the ability of a number of possible intermediates to replace Z for growth, we should, and often do, obtain valuable information as to the course of the synthesis.

This approach is obviously more satisfactory when we can use the same species of organism throughout; this can be done in two ways. First, we may use the biochemical mutants; a number of strains with independent mutations leading to loss of ability to synthesize a particular metabolite are often available. If the test organism has a sexual phase in the life cycle, it is also usually possible to determine which of the mutants differ from one another genetically and are likely to have failed at different steps of the synthesis. Secondly, we could also get results with a single species by using the inhibitory analogue technique for inducing failure to produce necessary coenzymes for different steps in the sequence of reactions; this approach has been little used so far.

Excretion or accumulation of intermediates. One difficulty of all these methods of approach is to decide, other than by intelligent chemical guesswork, which substances to try as possible intermediates. But the organism itself often provides valuable aid.

In all the types of metabolic lesion which have just been discussed it may happen that, when the final metabolite is provided so that the organism can grow, the intermediate which cannot be metabolized further continues to be synthesized; this intermediate (or a secondary product derived from it) accumulates in the cell and is often excreted into the culture medium. The isolation of such material has sometimes led to the identification of quite unsuspected intermediates. Such accumulations have been found frequently with biochemical mutants (e.g. W and X with mutants m_2 and m_3 in the scheme of Fig. 2); it has also occurred (e.g. B) when a reaction is prevented by the functional lack of a coenzyme, and again when the utilization of an intermediate (A) is prevented by an analogue (A^1).

That such excretion is occurring may be first indicated by the ability of the metabolic products of one strain to support the growth of another with a metabolic lesion at an earlier stage; thus in the scheme of Fig. 2 the mutant m_2 excreting W would support the growth of mutant m_1 which is blocked at the previous stage.

Simultaneous adaptation

A further method of detecting intermediates rests on the abolition of an existing lesion rather than the creation of a new one. Suppose that an organism does not normally metabolize N (Fig. 2), but forms an adaptive enzyme for doing so when N is present, the ultimate product being Q. Then it follows that, unless the organism normally has the power to convert O to P and P to Q, then adaptation to metabolism of N must also have caused adaptation to O and P. This can be tested with appropriate controls. The technique has been developed particularly by Stanier for the study of catabolic pathways, but it has not yet been possible to use it much in studies of biosynthesis.

Necessity for supporting evidence

The interpretation of results obtained by these various methods of approach is unfortunately rarely quite so simple and clear-cut as has been indicated in the formalized examples given. This makes it especially important that such results should be regarded as a signpost to future work and not as a proof of the mechanisms indicated.* Furthermore, since all synthetic reactions must require at least two substrates (e.g. Y and Q giving rise to Z), information about the other component may often still be lacking.

---

* Mitchell (1953) has recently particularly emphasized this point in connexion with work with biochemical mutants.

We must therefore seek supporting evidence at other levels of investigation, and the use of cell suspensions for studying the metabolism of defined substrates is clearly indicated.

Metabolism of cells deficient in a growth factor. Cell suspensions or tissues deficient in a given vitamin or growth factor have long been employed in attempts to locate the metabolic function of such metabolites. The technique was first used by Lwoff (1934) in the case of micro-organisms and by Peters & Thompson (1934) with animal tissues. The cells are harvested from media containing only sub-optimal concentrations of the growth factor and their metabolism compared in detail with that of normal cells. Any difference found may indicate reactions in which the factor has a function. Previous work with the growing organism may fortify this approach. Firstly, we can now, in some cases, grow cells in good yield in the complete absence of the growth factor by making use of appropriate replacement mixtures; we thus obtain totally instead of partially deficient cells. Secondly, we may obtain hints from replacement studies as to the type of metabolic pathway in which the factor has coenzyme function and even as to the actual step in the sequence of reactions. Thus if C but not B (in Fig. 2) will replace F for growth then F probably functions in the conversion of B to C.

General

The methods of approach to research on biosynthetic pathways which I have tried to piece together in some sort of co-ordinated way arose, of course, from individual experimental observations and their interpretation and confirmation. The realization of their value has come not from theoretical discussion but from frequent use in practice. Some of these observations are milestones in the progress of chemical microbiology; if there had been time it would certainly have been more instructive to follow the development of these ideas rather than to give, as I have had to do, merely a cross-section in very general terms.

In the time which remains a few specific examples will be considered which illustrate a number of these points.

The experimental work on which the preceding discussion is based, the deductions made therefrom, and the difficulties of interpretation which may arise are in the main covered by the following reviews (among others) to whose authors (except himself), and all the workers whose experiments and ideas are included, the lecturer pays grateful tribute:

General nutrition and metabolic function: Lwoff (1944), Knight (1945), Gunsalus (1949), Snell (1951*a*, *b*, 1952), Woods (1950*b*, 1952*a*).

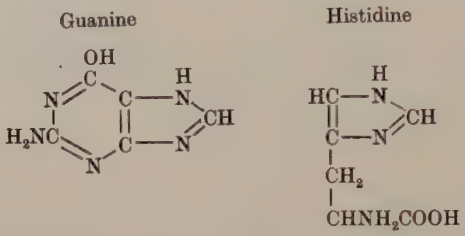
Inhibitory analogues of metabolites: Roblin (1946), Fildes (1948), Woolley (1952), Shive (1950, 1952).

Biochemical mutants: Beadle (1945), Mitchell (1950), Emerson (1950), Catcheside (1951), Davis (1950, 1952*a*).

Simultaneous adaptation: Stanier (1950, 1951).

Relationship between purine and histidine

One other type of observation springing from quantitative studies of bacterial nutrition may yield valuable initial information. Suppose that a metabolite such as C (Fig. 2) is required both directly for incorporation into a cell constituent and also as precursor of another such compound D. Then we should expect that the quantitative requirement for C should be less if D is provided preformed in the medium. The work of Snell, who has made so many brilliant contributions to bacterial nutrition, provides an example of this. A strain of *Lactobacillus casei* was found which required guanine for growth, but was able to synthesize histidine. The chemical structures of the two compounds (Fig. 3) suggested to Broquist & Snell (1949) that one might be the precursor of the other, and they found in fact that the requirement for purine was halved by the concurrent presence of histidine (Fig. 3).



*Lb. casei**	Guanine required for half-maximum growth µg./ml.
Histidine absent	11
Histidine present	5

* Strain synthesizing histidine under conditions where purine is required for growth.

Fig. 3. The relationship of guanine to histidine. Data interpreted from graphs given by Broquist & Snell (1949).

The function in metabolism of folic acid and its derivatives

The realization of the value of the growth factor replacement technique and the use of inhibitory analogues owes much to the practical help it has given in elucidating the function in the cell of the folic acid group of growth factors. The chemical structures of some of the members of this group are given in Fig. 4; I shall use the term folic acid in general reference to the more complex members of the group, i.e. those containing the pteridine residue, and refer to specific members by the names shown in the figure. Without burdening you with too much chemistry and details of the nutritional requirements of a multiplicity of organisms, the points I wish to make are as follows: (*a*) all these substances are active with one organism or another as the minimal requirement (within the group) for growth, (*b*) all of them contain a *p*-aminobenzoic acid residue; the latter is itself active with some organisms which when given it can complete the synthesis of folic acid, and (*c*) some form of folic acid, probably more complex in structure than any of those already known, is likely to be the ultimate coenzyme form of the group.

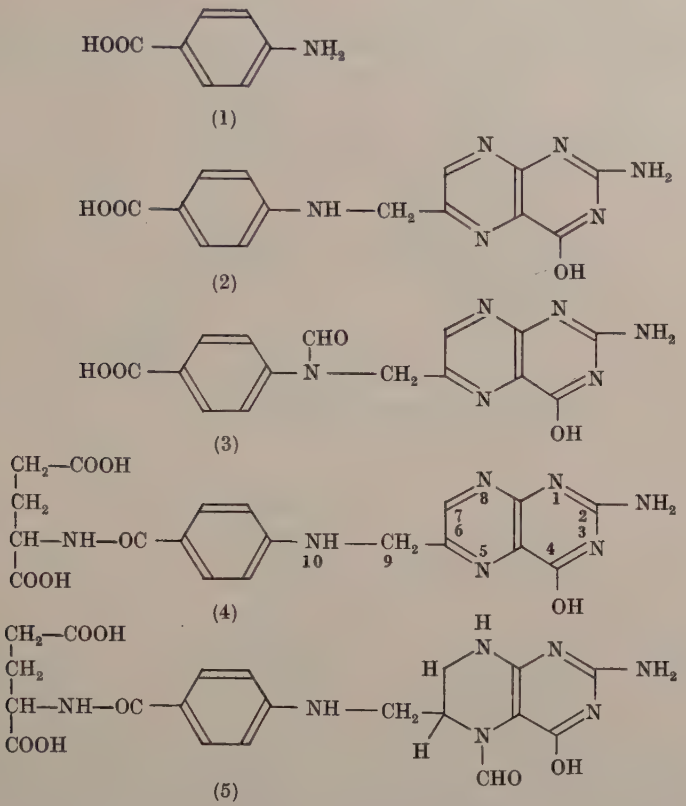

HOOC
NH₂
(1)
HOOC
NH—CH₂
N
N
NH₂
N
N
OH
(2)
CHO
HOOC
N—CH₂
N
N
NH₂
N
N
OH
(3)
CH₂—COOH
CH₂
CH—NH—OC
COOH
NH—CH₂
10
9
N
N
8
1
7
2
6
3
5
4
NH₂
N
N
OH
(4)
CH₂—COOH
CH₂
CH—NH—OC
COOH
NH—CH₂
H
N
H
N
NH₂
H
N
N
CHO
OH
(5)

Fig. 4. The structure of some members of the folic acid group. (1) *p*-Aminobenzoic acid; (2) pteroic acid; (3) rhizopterin; (4) pteroylglutamic acid; (5) N^5-formyltetrahydropteroylglutamic acid (probable structure of leucovorin and folinic acid—SF).

It is now generally accepted that the sulphonamides owe their growth-inhibitory property to the fact that they are analogues of *p*-aminobenzoic acid, which is an essential metabolite. *p*-Aminobenzoic acid was in fact first discovered as a substance of biological interest through its ability to overcome sulphonamide inhibition of growth in a competitive manner. In the present sense therefore the sulphonamides may be used as tools for creating an effective deficiency of *p*-aminobenzoic acid, and thus of folic acid, in the systems under test.

The metabolic inter-relationships of *p*-aminobenzoic acid and the various members of the folic acid group are too complex to deal with in detail here. Briefly, experiments with growing cultures and cell suspensions of the kind which I have outlined support the view, first, that all the known functions of *p*-aminobenzoic acid are exerted through its conversion to a coenzyme form of folic acid, and, secondly, that none of the present known forms of folic acid is the actual coenzyme (or prosthetic group) of the enzymes concerned.

The ability of mixtures of substances (Fig. 5) of quite different chemical type to folic acid to replace *p*-aminobenzoic acid and folic acid for the growth of various organisms, and to overcome inhibition of growth by sulphonamides and analogues of folic acid, gave the first information as to the probable function of folic acid. These substances (certain nucleic acid derivatives, certain amino-acids, vitamin B_{12}) are therefore, by the argument advanced earlier, probable products of the coenzyme-like function of folic acid.

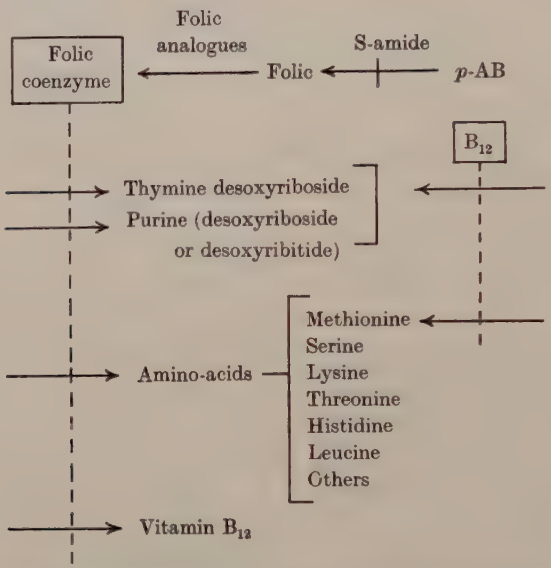


Fig. 5. Scheme showing probable relationship between *p*-aminobenzoic acid, folic acid and the products of their function in metabolism.

These apparent products are many and varied in chemical structure. This suggests that there is some common type of chemical change involving the same coenzyme at some stage of the synthesis of each. As far as has yet been investigated this has proved to be the case, but not all of the specific reactions in the individual synthetic pathways have yet been tracked down and the requirement for folic acid fully established.

In the case of serine and methionine further 'replacement' experiments (see p. 157) with known possible primary intermediates have enabled the particular reaction to be located with some certainty. Thus with serine it is the condensation of glycine with a single-carbon residue, and with methionine the final methylation of homocysteine (Fig. 6). In purine synthesis it was an excretion which gave the first clue. Cultures of *Bact. coli* with restricted available *p*-aminobenzoic acid or folic acid were found to excrete a diamine, first identified as 4-aminoimidazole-5-carboxamide (see Shive, 1950) and very recently shown by gentler methods of isolation to be a riboside derivative of the amine (Greenberg, 1952). Its possible conversion to purine riboside by incorporation of a single-carbon unit and ring closure is easy to see (Fig. 6).

In our laboratory we* have tried to confirm and extend some of these results at the cell suspension level of investigation. Harvested cells deficient in members of the folic acid group were obtained from growth on suitable replacement mixtures. Synthesis of serine was obtained with suspensions of various organisms in the presence of glycine, formate and glucose but only if *p*-aminobenzoic acid or folic acid (depending on the organism) were added (Fig. 6). In similar experiments synthesis of methionine from homocysteine by various mutants of *Bact. coli* was found to require both *p*-AB and B_{12}; there was strong evidence that under the test conditions serine is the donor of the required one-carbon unit.

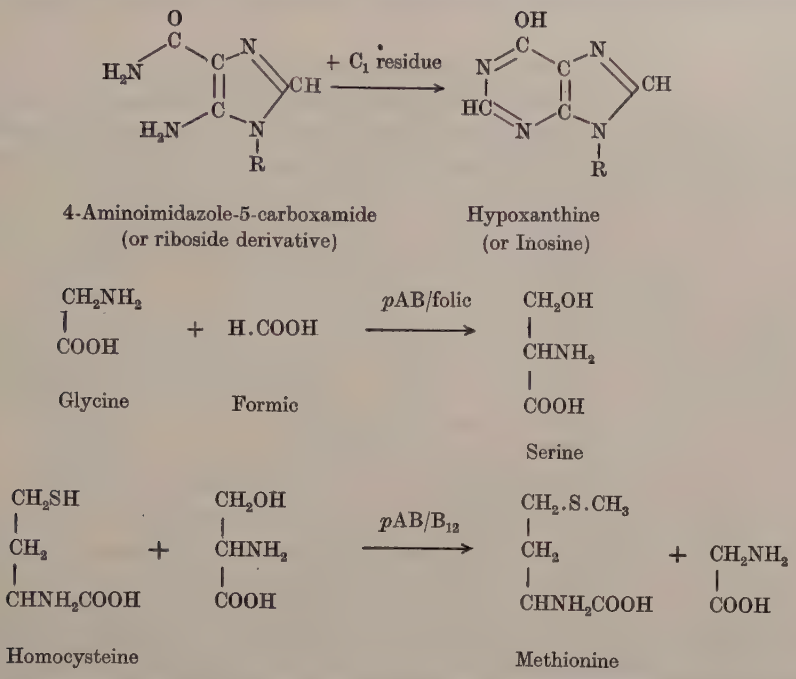


Fig. 6. Some reactions in which members of the folic acid group have coenzyme-like function.

In all three reactions shown in Fig. 6 there is a condensation of a one-carbon unit with the other substrate, and all lead to compounds which are probable products of the function of folic acid. It was first suggested by Shive (1950) and is now a generally accepted hypothesis, that the coenzyme form of folic acid, whatever it may turn out to be chemically, is concerned in the metabolic transfer of single-carbon residues.

It seems likely that further details concerning the coenzyme-like function of folic acid and its mechanism will require suitable systems for investigation at the cell-free enzyme preparation level.

---

* In collaboration with J. Lascelles, F. Gibson, M. J. Cross, P. M. Meadow and S. Wijesundera.

The experimental work and ideas which have been summarized in a rather general way in this section of the lecture are derived from literally hundreds of papers to which it has unfortunately been impossible to make detailed reference. Full references and detailed discussion will be found in the following reviews and papers: Jukes & Stokstad (1948), Hutchings & Mowat (1948), Winkler & de Haan (1948), Shive (1950, 1951), Woolley (1952), Welch & Nichol (1952), Woods (1952*a*, *b*, 1953).

Biosynthesis of aromatic compounds

The mention of the word 'organic' would, I suppose, bring most frequently to the mind's eye of a microbiologist the picture of a benzene ring. Yet until recently very little was known about the synthesis by living organisms of this simplest of the aromatic structures.

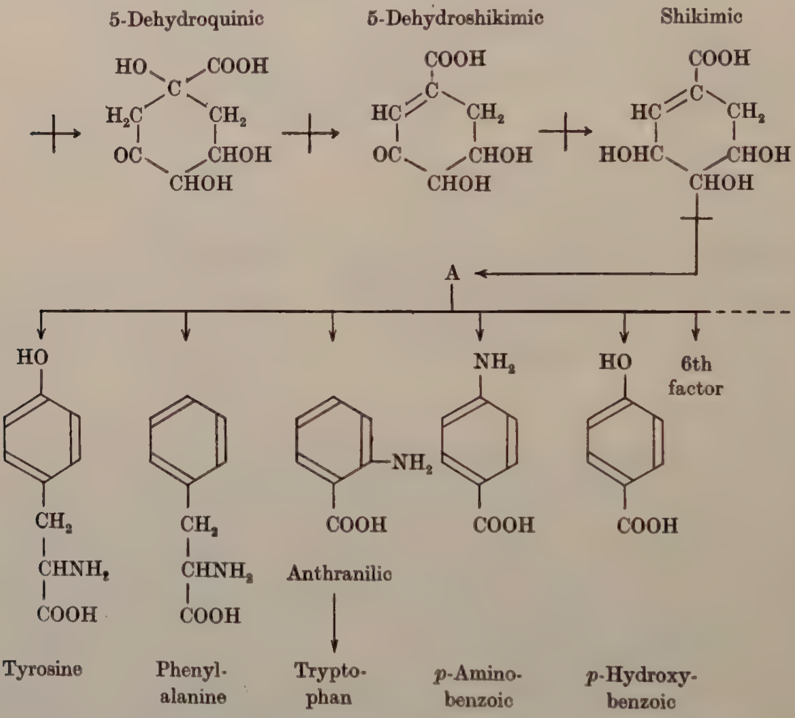


Fig. 7. Stages in the biosynthesis of some aromatic compounds based on the scheme of Davis (1952*a*).

I will take as an example of the value of nutritional studies with biochemical mutants some recent work of Davis on the bacterial synthesis of a number of aromatic compounds (for references see Davis, 1950; 1952*a*, *b*, *c*).

Making use of the ingenious penicillin technique devised simultaneously by himself and by Lederberg & Zinder (1948), Davis isolated a number of mutants of *Bact. coli* with multiple requirements for the five aromatic compounds shown in the lower half of Fig. 7. Various mutants required two, three, four or all five of this group; the combinations of requirements were always for successive compounds in the order shown (from left to right) and starting with tyrosine. It was unlikely from the frequency of their occurrence, and other evidence, that these strains had arisen as a result of multiple mutations at different loci controlling steps in independent synthetic pathways leading to the five aromatics. It was more likely that the formation of a common precursor (e.g. A in Fig. 7) or precursors of the successive groups of substances had failed.

Search was therefore made among a great variety of aromatic compounds and cyclohexane derivatives for a substance which could promote growth in the absence of some or all of the five compounds. Eventually Stanier, who was also a collector of such compounds for his work by the simultaneous adaptation technique on the oxidation of aromatic substances (see Stanier, 1950), suggested and provided a sample of shikimic acid. This was active with a number (though not all) of the mutants in totally replacing the aromatic requirements; it was probably, therefore, a common precursor of them all.

The role of shikimic acid as an intermediate in aromatic biosynthesis was confirmed by the fact that some of the mutants which do not respond to shikimic acid, and are presumably unable to utilize it, were found to excrete it in considerable quantity. Other mutants of this series were found to excrete different substances; this was detected in the first place by the ability of some mutants to support the growth of others. The identification of these compounds as dehydroshikimic acid and dehydroquinic acid enabled Davis to propose the pathway shown in Fig. 7 for the earlier stages of aromatic synthesis. Great progress has therefore been made, though we are not yet back as far as an open chain precursor.

Further ingenious work (Davis, 1952*b*, *c*) has provided a satisfactory explanation of why not all the mutants require all the aromatic compounds for growth although the metabolic lesions (as indicated by excretions of the above intermediates) are all in the early common pathway. Strong evidence was obtained that a number of the mutants were not totally deficient in available shikimic acid, and that this substance was used preferentially for the synthesis of *p*-hydroxybenzoic acid, *p*-aminobenzoic acid and so on in decreasing order of priority. Dehydroshikimic acid itself was found to inhibit competitively the utilization of shikimic acid; in a mutant with metabolic lesions both before and after dehydroshikimic acid (so that it was no longer an effective metabolite) increasing concentrations of dehydroshikimic acid relative to shikimic acid induced growth requirements for tyrosine, then phenylalanine plus tyrosine, and so on in the usual combinations. These points illustrate also some of the difficulties which may arise in interpreting nutritional experiments with mutants.

Perhaps I may make a short diversion at a more macrobiological level on the other known biological occurrence of shikimic acid. The sample used initially by Davis had been isolated by H. O. L. Fischer from *Illicium religiosum*. Since this plant has such an enticing name (*ab illiciendo*) I have tried, with the aid of a botanical colleague, to find out something about it. It is a member of the order Magnoliaceae, and there is a delightful description of it by Hooker in Curtis's *Botanical Magazine* for 1843 (vol. XVI, p. 11)* together with a coloured engraving. After mentioning that this is the sacred aniseed tree of Japan, and that a fine young plant flowered in great profusion in March 1842 in the greenhouse at the Royal Botanic Gardens at Kew, Hooker goes on

> By the Japanese this plant is held sacred, they strew wreathes of it and branches over the tombs of their friends, and the priests burn the bark upon the altars of their deities. A singular use is made of the pulverized bark by the public watchmen. Hollow tubes graduated on the outside, are filled with this substance, which is lighted at one extremity, and burns gradually and uniformly: so that when the fire has reached a certain mark, the watchmen strike the hour upon a bell, and thus announce it to the public.

It seems that perhaps after all Bernard Davis was not the first person to ring a bell with shikimic acid!

Pyruvate oxidation factor

My last main example concerns some work which seems to me to illustrate most admirably the interplay of research at the levels of nutrition and metabolism resulting in great progress towards a full solution of the problem.† Furthermore, one phase of these investigations was initiated (see Gunsalus, 1949) with the intention of applying such concepts.

Pyruvate is properly regarded as one of the key intermediates in the metabolism of all types of cell, and thiamine pyrophosphate (aneurin diphosphate) is well known as coenzyme in a number of its metabolic transformations. One such reaction brought about by bacterial as well as animal cells is its oxidative decarboxylation to acetate and CO_2 (Fig. 8). Gunsalus, O'Kane and their colleagues found that cells of a strain (10 C 1) of *Strep. faecalis* harvested from a complex medium had high activity in this reaction. But cells harvested from a chemically defined medium, which supported good growth, were almost inactive. The activity of the latter suspensions was restored by the addition of yeast extract, but not by any of the known vitamins. It appeared, therefore, that some new and unknown coenzyme-like factor was concerned in the oxidation of pyruvate by this organism, and it was named pyruvate oxidation factor (P.O.F.) (O'Kane & Gunsalus, 1948; O'Kane, 1950; Gunsalus, Dolin & Struglia, 1952; Gunsalus, Struglia & O'Kane, 1952). Highly active concentrates from yeast extract were obtained.

Meanwhile work had been in progress in other laboratories on two unidentified growth factors for micro-organisms (Fig. 8). First, Snell and his colleagues had found that acetate stimulated the early growth of several lactobacilli and could be replaced by minute amounts of yeast extract (Guirard, Snell & Williams, 1946*a*, *b*). Secondly, a factor (later called protogen) present in liver and required for the growth of the ciliate *Tetrahymena geleii* W had been discovered by Kidder and his group (see Kidder & Dewey, 1949, 1951) and its concentration and isolation pursued by the Lederle group of workers (Stokstad, Hoffmann, Regan, Fordham & Jukes, 1949). It became clear that these two factors had similar properties both to one another and to pyruvate oxidation factor; they were found to be largely interchangeable in their biological effects (Snell & Broquist, 1949; Reed, De Busk, Gunsalus & Hornberger, 1951).

---

* I am much indebted to Dr J. H. Burnett of the Department of Botany, Oxford, for tracing this reference.

† Since much of this work is rather recent, and there are no comprehensive reviews, references to the original literature will be given in this section.

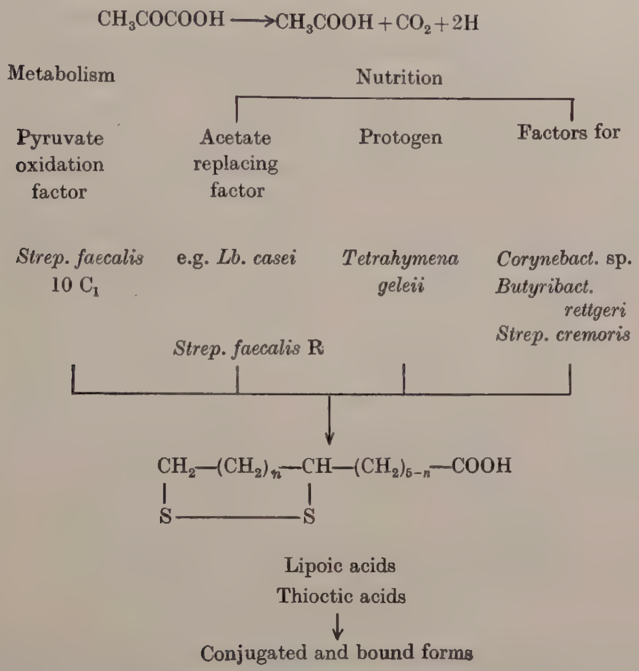

CH₃COCOOH → CH₃COOH + CO₂ + 2H
Metabolism
Nutrition
Pyruvate oxidation factor
Acetate replacing factor
Protogen
Factors for
Strep. faecalis 10 C₁
e.g. Lb. casei
Tetrahymena geleii
Corynebact. sp.
Butyribact. rettgeri
Strep. cremoris
Strep. faecalis R
CH₂—(CH₂)ₙ—CH—(CH₂)₅₋ₙ—COOH
S——S
Lipoic acids
Thioctic acids
Conjugated and bound forms

Fig. 8. Scheme illustrating the relationship and biological activities of pyruvate oxidation factor and related growth factors.

While these factors were being purified several other organisms (listed in Fig. 8) were found to require the new vitamin for growth (Stokstad, Hoffmann & Belt, 1950; Lytle & O'Kane, 1951; Kline, Pine, Gunsalus & Barker, 1952).

Two groups of workers (Gunsalus, Reed and their colleagues and the Lederle team) each succeeded in isolating crystalline material from natural sources. They were in pursuit respectively of pyruvate oxidation factor and protogen, but each used a variety of the biological effects (Fig. 8) in their test systems. They called their products α-lipoic acid and protogen B (see Reed, Gunsalus, Schnackenberg, Soper, Boaz, Kern & Parke, 1953; Patterson, Brockman, Day, Pierce, Macchi, Hoffmann, Fong, Stokstad & Jukes, 1951). Examination of the chemical properties, and finally the synthesis of active material, showed that the structure was that of a dimercapto-n-octanoic acid (Fig. 8), but the exact position of the sulphur atoms is not yet established. The groups of active synthetic compounds are referred to as synthetic lipoic acids and thioctic acids respectively (Hornberger, Heitmiller, Gunsalus, Schnackenberg & Reed, 1953; Bullock, Brockman, Patterson, Pierce & Stokstad, 1952). Conjugated or bound forms exist in nature from which the new factor is released by acidic or enzyme hydrolysis (Reed, De Busk, Johnston & Getzendaner, 1951; Gunsalus, Struglia & O'Kane, 1952).

Coenzyme form. It would be in accord with usual experience in this sort of work if the substance first recognized as a growth factor were a less elaborate molecule than the ultimate coenzyme form (as, for example, with pyridoxin and *p*-aminobenzoic acid). Furthermore, an organism might well be found which was unable to complete the synthesis of the more complex form (possibly one of the conjugates mentioned above) and required it as a growth factor.

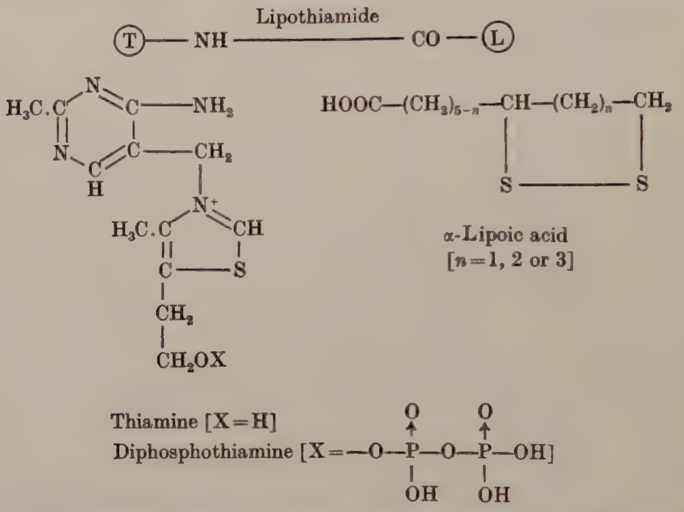


Fig. 9. The relation of lipothiamide and its pyrophosphate to α-lipoic acid and thiamine. Based on the work of Reed & De Busk (1952*b*).

Reed & De Busk (1952*a*, *b*) made a deliberate search for such an organism among mutants of *Bact. coli* responding to yeast extract. They found one which would not grow with free α-lipoic acid, but which did so when supplied with one of the complex forms with activity as acetate-replacing factor for *Strep. lactis*. They had therefore a very useful tool for further investigations of the nature of the higher form.

The next step was to identify the other part or parts of the active conjugate molecule. Thiamine (Fig. 9) was a possibility in view of its known role in pyruvate metabolism. Again at the level of growing cultures Reed & De Busk (1952*b*) found that the addition of acetate to the medium for *Strep. lactis* not only abolished the need for α-lipoic acid (as expected) but also that for thiamine. It was likely therefore that acetate was, under the test conditions, a product of the joint function of the two growth factors. They next went to the cell suspension level and incubated harvested cells of the same organism with α-lipoic acid and thiamine; the reaction products supported the growth of the mutant strain of *Bact. coli* but only if both factors had been present during the incubation. *Streptococcus lactis* could therefore synthesize the form required by the mutant from α-lipoic acid and thiamine. Finally, Reed & De Busk obtained material with similar activity by heating the two substances together *in vacuo* at 130° and by other methods. Chemical evidence showed an amide link between the two molecules and the structure indicated in Fig. 9 was proposed; the compound was called lipothiamide.

The function of lipothiamide in pyruvate metabolism of *Bact. coli* was shown by the use of cell suspensions of the mutant deficient in this factor. These were obtained from cultures on a medium containing casein hydrolysate (which apparently supplies all the products of the coenzyme function), but no lipothiamide. The deficient cells had negligible pyruvate oxidase activity unless lipothiamide were added; the component vitamins, either alone or in admixture, were inactive.

Extending this work finally to the level of cell-free enzyme preparations, Reed & De Busk (1952*c*, 1953) have recently reported that the extracted apoenzyme (from deficient cells) could not be reactivated even by lipothiamide, but required the pyrophosphate ester (Fig. 9) which may therefore be the actual coenzyme.

In connexion with this work it is interesting to note that Peters, Stocken & Thompson (1945) had suspected some time earlier that a dithiol compound was a component of the pyruvate oxidase system of animal tissues from their work on the inhibition of this enzyme by trivalent arsenicals (such as lewisite), and the removal of such inhibition by the dithiol compound British anti-lewisite (BAL).

Some further considerations concerning metabolic function of growth factors

Even when a function has been discovered for a growth factor in some metabolic event within the cell it still does not prove that it is this reaction and function which is of critical importance for growth. This point has been well stressed recently by Snell (1952) who has shown actually in experiments with growing cultures that several of the known reactions in amino-acid metabolism in which vitamin B_6 derivatives have coenzyme function are indeed important for growth.

It is also necessary to consider whether a B-group vitamin may not have other effects which are far from catalytic. Dr Moulder and I were recently surprised to find that the growth of a strain of yeast (*Saccharomyces cerevisiae* Y 47), with which we happened to be working, was potently inhibited by a mixture of adenine and nicotinic acid (Moulder & Woods, 1953). Both these compounds are certainly essential metabolites within the usual meaning of the word. Adenine is indeed required for the growth of this organism in the absence of *p*-aminobenzoic acid, while nicotinic acid (or a derivative) is demonstrably synthesized. The concentrations required for inhibition (Fig. 10) are only of the order required for growth by organisms which cannot synthesize them.

These results recalled to us a similar and even more striking case discovered by Rabinowitz & Snell (1951) with another species of yeast (*Saccharomyces carlsbergensis* 4228). Thiamine, which is not required as an exogenous growth factor, actually inhibited growth at concentrations (Fig. 10) which are certainly as low, if not lower, than those required by exacting organisms.

The inhibitions were in both cases overcome by traces of vitamin B_6 (Fig. 10); the latter is not normally required as a growth factor by either organism. So that the presence of the other metabolites had induced a requirement for vitamin B_6. This is reminiscent of the inhibitory interrelationships between amino-acids first observed by Gladstone (1939) in studies of the nutrition of the anthrax bacillus.

	Growth inhibited 50 % by	Full growth restored by
Sacch. carlsbergensis 4228	10^{-10} M-thiamine	10^{-10} M-pyridoxal
Sacch. cerevisiae Y47	10^{-6} M-adenine and 10^{-6} M-nicotinic acid	10^{-9} M-pyridoxin

Neither yeast requires pyridoxal or pyridoxin for full growth on its basal medium.

Fig. 10. Growth factors as inhibitors of the growth of yeast. Data obtained from Rabinowitz & Snell (1951) and Moulder & Woods (1953).

There is no explanation yet to hand for these inhibitory effects of vitamin B-group substances on the growth of yeast, but it certainly seems that substances known as growth factors may have, at least in growing cultures, quite different effects to those discussed earlier in the lecture. Perhaps such effects may play a part in the co-ordinated control of interwoven metabolic pathways.

A recapitulation

Lest we become a little too complacent after reviewing some of the achievements of biochemical microbiology, let us examine again some of the methods we have used to attain our ends.

To begin with there is certainly no doubt that the microbes worked with by biochemical microbiologists must be considered a privileged class! To study their growth, or to obtain our cell suspensions, we normally put them into a rich environment, which, whether chemically defined or not, contains an abundance of everything we think they may want. Here they live a life of ease and luxury for perhaps twenty cell generations, during which time mutants may be selected (or the cells adapt enzymically) so that they are specially suited to this comfortable life. Have we any right to assume that by studying them we can get a true picture of the life of their working class cousins in, let us say, the soil? It seems that long ago Winogradsky had similar misgivings in the realms of purer microbiology (see Stanier, 1953; Winogradsky, 1949).

Perhaps I may quote one example from our own recent experience. My colleague Wijesundera and I were extending to other strains of *Bact. coli* some work of this Unit on the synthesis of methionine from homocysteine by cell suspensions of this organism. A strain requiring p-aminobenzoic acid was grown in a replacement mixture (containing methionine) in order to obtain cells deficient in the vitamin. One strain, tested after harvesting from such a medium, synthesized almost no methionine, whereas when it was grown in the absence of methionine (presence of p-aminobenzoic acid) it synthesized well. The effect was found to be due to methionine itself and was proportional to the methionine concentration during growth (Wijesundera & Woods, 1953). A single overnight sub-cultivation in the presence of methionine had led to an almost complete loss of ability to synthesize this amino-acid. A similar effect, of a considerable though variable extent, was found with a number of other strains of the organism. Cohn, Cohen & Monod (1953), who were studying methionine synthesis from a rather different point of view, have concurrently observed the same phenomenon.

In the preface to the first edition of her book *Bacterial Metabolism*, Marjory Stephenson (1930) surveyed the general position and methods of investigation in our subject in 1930. She then wrote

> We are indeed in much the same position as an observer trying to gain an idea of the life of a household by a careful scrutiny of the persons or material arriving at or leaving the house: we keep accurate record of the foods and commodities left at the door and patiently examine the contents of the dustbin and endeavour to deduce from such data the events occurring within the closed doors.

What is the position now after a further twenty-three years? I do not think that we have yet succeeded in opening the door and paying a friendly visit. But we have certainly devised some ingenious methods of what I can only call 'peep-tomming'—or should it be 'tom-peeping'? These methods are, however, strangely reminiscent of the atomic age that grew up parallel with them. We subject the inhabitants of the house to noxious radiations and mustard gas and cause them to mutate. We shatter and disintegrate the house by violent physical means, and we study the debris on the lawn and the feeble and unco-ordinated flutterings of such signs of life as we find. We blockade the house and starve the inhabitants by preventing the delivery of essential foodstuffs. Worse still, we subtly fashion loaves of clay and bribe the baker's boy to deliver them instead of bread! For this ultimate beastliness, 'this further innovation in cell torture' as Marjory Stephenson (1946) once called it in referring to some work of my other chief Sir Paul Fildes (1941), I suppose that I must myself bear a large measure of responsibility.

Perhaps it is, after all, not without reason that our well-meant efforts are sometimes referred to as 'microbating' by our more biological colleagues.

By all this my real intention is to renew the plea that we should check our results, whenever possible, at the more biological end of our spectrum of methods of investigation.

If I have concluded this lecture on a lighter note, it is only because my long association with 'M.S.' (as she was always affectionately known to us) taught me clearly that it was equally part of her philosophy that in chemical microbiology there should be some measure of gaiety as well as good science.

This lecture is dedicated, in all humility, to the memory of the late Dr Marjory Stephenson, F.R.S.

I am deeply conscious of the debt that I owe, not only to her, but also to Sir Paul Fildes, F.R.S. and Sir Rudolph Peters, F.R.S. for all that they have made known to me of the deeper meaning of microbiology and biochemistry. To many colleagues in this and other lands, and especially to Dr D. W. Woolley, I am indebted for fruitful discussion of some of the subjects considered in this lecture. Finally, I acknowledge with gratitude the willing help given by my colleagues in the Microbiology Unit of this Department both in assembling the material and debating some of the points which arose.

REFERENCES

Anonymous (1945). Society for General Microbiology. *Nature, Lond.* **155**, 796.

Beadle, G. W. (1945). Genetics and metabolism in Neurospora. *Physiol. Rev.* **25**, 643.

Broquist, H. P. & Snell, E. E. (1949). Studies on the mechanism of histidine synthesis in lactic acid bacteria. *J. biol. Chem.* **180**, 59.

Bullock, M. W., Brockman, J. A., Patterson, E. L., Pierce, J. V. & Stokstad, E. L. R. (1952). Synthesis of DL-thioctic acid. *J. Amer. chem. Soc.* **74**, 1868.

Catcheside, D. G. (1951). *The Genetics of Micro-organisms.* London: Sir Isaac Pitman and Sons, Ltd.

Cohn, M., Cohen, G. N. & Monod, J. (1953). L'effet inhibiteur spécifique de la méthionine dans la formation de la méthionine-synthèse chez *Escherichia coli. C.R. Acad. Sci., Paris*, **236**, 746.

Davis, B. D. (1950). Studies on nutritionally deficient bacterial mutants isolated by means of penicillin. *Experientia*, **6**, 41.

Davis, B. D. (1952*a*). Intermediates in the biosynthesis of proline, ornithine, histidine, lysine and the aromatic amino acids. Symposium sur le Métabolisme Microbien, p. 32. *Int. Congr. Biochem.* 2. Paris, 1952.

Davis, B. D. (1952*b*). Aromatic biosynthesis. IV. Preferential conversion, in incompletely blocked mutants, of a common precursor of several metabolites. *J. Bact.* **64**, 729.

Davis, B. D. (1952*c*). Aromatic biosynthesis. V. Antagonism between shikimic acid and its precursor, 5-dehydroshikimic acid. *J. Bact.* **64**, 749.

Elsden, S. R. & Pirie, N. W. (1949). Obituary notice. Marjory Stephenson, 1885–1948. *J. gen. Microbiol.* **3**, 329.

Emerson, S. (1950). Competitive reactions and antagonisms in the biosynthesis of amino acids by Neurospora. *Cold Spr. Harb. Sym. quant. Biol.* **14**, 40.

Fildes, P. (1941). Inhibition of bacterial growth by indole acrylic acid and its relation to tryptophan; an illustration of the inhibitory action of substances chemically related to an essential metabolite. *Brit. J. exp. Path.* **22**, 293.

Fildes, P. (1948). Planned chemotherapy. *Irish J. med. Sci.* p. 659.

Gladstone, G. P. (1939). Inter-relationships between amino-acids in the nutrition of *B. anthracis. Brit. J. exp. Path.* **20**, 189.

Greenberg, G. R. (1952). Isolation of 4-amino-5-imidazole-carboxamide riboside from the culture medium of sulphonamide-inhibited *Escherichia coli. J. Amer. chem. Soc.* **74**, 6307.

Guirard, B. M., Snell, E. E. & Williams, R. J. (1946*a*). Nutritional role of acetate for lactic acid bacteria. I. Responses to substances related to acetate. *Arch. Biochem.* **9**, 361.

Guirard, B. M., Snell, E. E. & Williams, R. J. (1946*b*). The nutritional role of acetate for lactic acid bacteria. II. Fractionation of extracts of natural materials. *Arch. Biochem.* **9**, 381.

Gunsalus, I. C. (1949). Comparative metabolism: bacterial nutrition and metabolic function. *Harvey Lect.* 1949–1950, 40.

Gunsalus, I. C., Dolin, M. I. & Struglia, L. (1952). Pyruvic acid metabolism. III. A manometric assay for pyruvate oxidation factor. *J. biol. Chem.* **194**, 849.

Gunsalus, I. C., Struglia, L. & O'Kane, D. J. (1952). Pyruvic acid metabolism. IV. Occurrence, properties and partial purification of pyruvate oxidation factor. *J. biol. Chem.* **194**, 859.

Hornberger, C. S., Heitmiller, R. F., Gunsalus, I. C., Schnackenberg, G. H. F. & Reed, L. J. (1953). Synthesis of DL-α-lipoic acid. *J. Amer. chem. Soc.* **75**, 1273.

Hutchings, B. L. & Mowat, J. H. (1948). The chemistry and biological action of pteroylglutamic acid and related compounds. *Vitam. & Horm.* **6**, 1.

Jukes, T. H. & Stokstad, E. L. R. (1948). Pteroylglutamic acid and related compounds. *Physiol. Rev.* **28**, 51.

Kidder, G. W. & Dewey, V. C. (1949). Studies in the biochemistry of *Tetrahymena.* XI. Components of factor II of known chemical nature. *Arch. Biochem.* **20**, 433.

Kidder, G. W. & Dewey, V. C. (1951). The biochemistry of ciliates in pure culture. *Biochemistry and Physiology of Protozoa* (ed. by A. Lwoff), p. 323. New York: Academic Press, Inc.

Kline, L., Pine, L., Gunsalus, I. C. & Barker, H. A. (1952). Probable identity of the growth promoting factor for *Butyribacterium rettgeri* with other biologically-active substances. *J. Bact.* **64**, 467.

Knight, B. C. J. G. (1945). Growth factors in microbiology. Some wider aspects of nutritional studies with micro-organisms. *Vitam. & Horm.* **3**, 105.

Lederberg, J. & Zinder, N. J. (1948). Concentration of biochemical mutants of bacteria with penicillin. *J. Amer. chem. Soc.* **70**, 4267.

Lwoff, A. (1934). Die Bedeutung des Blutfarbstoffes für die parasitischen Flagellaten. *Zbl. Bakt.* (1 Abt.), **130**, 497.

Lwoff, A. (1944). *L'Évolution Physiologique. Étude des Pertes de Fonctions chez les Microorganismes.* Paris: Hermann et Cie.

Lytle, V. L. & O'Kane, D. J. (1951). The pyruvate oxidation factor requirement of *Streptococcus cremoris. J. Bact.* **61**, 240.

Mitchell, H. K. (1950). Vitamins and metabolism in Neurospora. *Vitam. & Horm.* **8**, 127.

Mitchell, H. K. (1953). Growth factors in relation to studies of the genetics of microorganisms. Symposium on Nutrition and Growth Factors. *Congr. Int. Microbiol.* **6**. Rome, 1953.

Moulder, J. W. & Woods, D. D. (1953). Some inter-relationships between nicotinic acid, pyridoxin and purine in the growth of a strain of *Saccharomyces cerevisiae. J. gen. Microbiol.* **9**, iv.

O'Kane, D. J. (1950). Influence of the pyruvate oxidation factor on the oxidative metabolism of glucose by *Streptococcus faecalis. J. Bact.* **60**, 449.

O'Kane, D. J. & Gunsalus, I. C. (1948). Pyruvic acid metabolism. A factor required for oxidation by *Streptococcus faecalis. J. Bact.* **56**, 499.

Patterson, E. L., Brockman, J. A., Day, F. P., Pierce, J. V., Macchi, M. E., Hoffmann, C. E., Fong, C. T. O., Stokstad, E. L. R. & Jukes, T. H. (1951). Crystallization of a derivative of protogen-B. *J. Amer. chem. Soc.* **73**, 5919.

Peters, R. A. & Thompson, R. H. S. (1934). Pyruvic acid as an intermediary metabolite in the brain tissue of avitaminous and normal pigeons. *Biochem. J.* **28**, 916.

Peters, R. A., Stocken, L. A. & Thompson, R. H. S. (1945). British anti-lewisite (BAL). *Nature, Lond.* **156**, 616.

Rabinowitz, J. C. & Snell, E. E. (1951). The nature of the requirement of *Saccharomyces carlsbergensis* for vitamin B_6. *Arch. Biochem. Biophys.* **33**, 472.

Reed, L. J. & De Busk, B. G. (1952*a*). A conjugate of α-lipoic acid required for oxidation of pyruvate and α-ketoglutarate by an *Escherichia coli* mutant. *J. biol. Chem.* **199**, 873.

Reed, L. J. & De Busk, B. G. (1952*b*). Chemical nature of an α-lipoic acid conjugate required for oxidation of pyruvate and α-ketoglutarate by an *Escherichia coli* mutant. *J. biol. Chem.* **199**, 881.

Reed, L. J. & De Busk, B. G. (1952*c*). Lipothiamide pyrophosphate—coenzyme for oxidative decarboxylation of α-keto acids. *J. Amer. chem. Soc.* **74**, 3964.

Reed, L. J. & De Busk, B. G. (1953). Mechanism of enzymatic oxidative decarboxylation of pyruvate. *J. Amer. chem. Soc.* **75**, 1261.

Reed, L. J., De Busk, B. G., Gunsalus, I. C. & Hornberger, C. S. (1951). Crystalline α-lipoic acid: a catalytic agent associated with pyruvate dehydrogenase. *Science*, **114**, 93.

Reed, L. J., De Busk, B. G., Johnston, P. M. & Getzendaner, M. E. (1951). Acetate-replacing factors for lactic acid bacteria. I. Nature, extraction and distribution. *J. biol. Chem.* **192**, 851.

Reed, L. J., Gunsalus, I. C., Schnackenberg, G. H. F., Soper, Q. F., Boaz, H. E., Kern, S. F. & Parke, T. V. (1953). Isolation, characterization and structure of α-lipoic acid. *J. Amer. chem. Soc.* **75**, 1267.

Roblin, R. O. (1946). Metabolite antagonists. *Chem. Rev.* **38**, 255.

Shive, W. W. (1950). The utilization of antimetabolites in the study of biochemical processes in living organisms. *Ann. N.Y. Acad. Sci.* **52**, 1212.

Shive, W. W. (1951). The functions of B-vitamins in the biosynthesis of purines and pyrimidines. *Vitam. & Horm.* **9**, 75.

Shive, W. W. (1952). Biological activities of metabolite analogues. *Annu. Rev. Microbiol.* **6**, 437.

Snell, E. E. (1951*a*). Bacterial nutrition—Chemical factors. *Bacterial Physiology* (ed. by C. H. Werkman and P. W. Wilson), ch. vii. New York: Academic Press, Inc.

Snell, E. E. (1951*b*). Growth factors in bacterial nutrition. *Plant Growth Substances* (ed. by F. Skoog), p. 431. Madison: University of Wisconsin Press.

Snell, E. E. (1952). A correlation of chemical, enzymatic and growth-promoting properties of vitamin B_6. Symposium sur le Métabolisme Microbien, p. 47. *Int. Congr. Biochem.* **2**. Paris, 1952.

Snell, E. E. & Broquist, H. P. (1949). On the probable identity of several unidentified growth factors. *Arch. Biochem.* **23**, 326.

Stanier, R. Y. (1950). Problems of bacterial oxidative metabolism. *Bact. Rev.* **14**, 179.

Stanier, R. Y. (1951). Enzymatic adaptation in bacteria. *Annu. Rev. Microbiol.* **5**, 35.

Stanier, R. Y. (1953). Adaptation, evolutionary and physiological: or Darwinism among the micro-organisms. Contribution to Symposium on Adaptation in Micro-organisms. *Symp. Soc. gen. Microbiol.* **3**. 1953.

Stephenson, M. (1930). *Bacterial Metabolism* (1st ed., 1930; 2nd ed., 1939; 3rd ed., 1949). London: Longmans, Green and Co.

Stephenson, M. (1946). 'The debt of biochemistry to microbiology'. *Congrès des Sciences Pastoriennes pour le Cinquantenaire de la Mort de Louis Pasteur.* Paris: Comité du Cinquantenaire, Union Nationale des Intellectuels.

Stokstad, E. L. R., Hoffmann, C. E. & Belt, M. (1950). Role of protogen in the nutrition of an unidentified Corynebacterium. *Proc. Soc. exp. Biol., N.Y.* **74**, 571.

Stokstad, E. L. R., Hoffmann, C. E., Regan, M. A., Fordham, D. & Jukes, T. H. (1949). Observations on an unknown growth factor for *Tetrahymena geleii. Arch. Biochem.* **20**, 75.

Various Authors (1953). Contributions to Symposium on Adaptation in Micro-organisms. *Symp. Soc. gen. Microbiol.* **3**. 1953.

WELCH, A. D. & NICHOL, C. A. (1952). Water-soluble vitamins concerned with one-carbon and two-carbon intermediates. *Annu. Rev. Biochem.* **21**, 633.

WIJESUNDERA, S. & WOODS, D. D. (1953). The effect of growth on a medium containing methionine on the synthesis of this amino-acid by *Bacterium coli*. *Biochem. J.* **55**, viii.

WINKLER, K. C. & DE HAAN, P. G. (1948). On the action of sulfanilamide. XII. A set of non-competitive sulfanilamide antagonists for *Escherichia coli*. *Arch. Biochem.* **18**, 97.

WINOGRADSKY, S. (1949). *Microbiologie du Sol. Œuvres complètes*, p. 841. Paris: Masson et Cie.

WOODS, D. D. (1950*a*). Obituary notice. Marjory Stephenson, 1885–1948. *Biochem. J.* **46**, 377.

WOODS, D. D. (1950*b*). Micro-organisms and vitamin research. *Research in Medical Science* (ed. by D. E. Green and W. E. Knox), p. 167. New York: The Macmillan Company.

WOODS, D. D. (1952*a*). Vitamin-B-group substances, especially folic acid and B_{12}, in the metabolism of micro-organisms. *Bull. World Hlth Org.* **6**, 35.

WOODS, D. D. (1952*b*). The function of folic acid and related growth factors in the metabolism of micro-organisms. Symposium sur le Métabolisme Microbien, p. 86. *Int. Congr. Biochem.* **2**. Paris, 1952.

WOODS, D. D. (1953). Folic acid and related compounds in the metabolism of micro-organisms. *Brit. med. Bull.* **9**, 122.

WOOLLEY, D. W. (1952). *A Study of Antimetabolites*. London: Chapman and Hall, Ltd.

(*Delivered before the Society for General Microbiology at its Sixteenth Meeting,* 13 *April* 1953)

Duguid, J. P. & Wilkinson, J. F. (1953). *J. gen. Microbiol.* 9, 174–189.

The Influence of Cultural Conditions on Polysaccharide Production by *Aerobacter aerogenes*

By J. P. DUGUID and J. F. WILKINSON
The Bacteriology Department, University of Edinburgh

SUMMARY: The polysaccharide content of *Aerobacter aerogenes* surface cultures grown on defined media was estimated by the anthrone method and related to the amount of growth as measured by the non-dialysable nitrogen content. Observations after different periods of incubation at 35° showed that on a medium containing excess sugar, growth was complete after 24 hr. and polysaccharide production was complete after 4 days. While most of the polysaccharide was produced after the cessation of growth, the rate of production per cell was greatest in the logarithmic phase. The polysaccharide : nitrogen ratio was low in 4-day cultures on media wherein cessation of growth was due to exhaustion of sugar (the sole carbon and energy source) and also was low in growths limited by anaerobic conditions or by the development of acidity. It was increased 10–20-fold in cultures wherein cessation of growth was due to exhaustion of limited nitrogen, phosphorus or sulphur sources, so that an excess of sugar remained available for extra polysaccharide synthesis. This great increase in the polysaccharide:nitrogen ratio of the culture was paralleled by similar increases in the degree of mucoidness as measured by the wet weight:nitrogen ratio, in the intracellular polysaccharide content as measured by periodate-Schiff staining, in capsular polysaccharide as measured by capsule diameter, and in loose slime polysaccharide as measured chemically in the supernatant after centrifugation of the culture. After 4 days at 35° the loose slime accounted for about half of the total polysaccharide in the highly mucoid cultures and about a fifth of that in the non-mucoid cultures.

The polysaccharides of bacteria can occur intracellularly, and extracellularly as capsules and loose slime. Intracellular polysaccharide is demonstrable microscopically by the specific periodate-Schiff staining method of Hotchkiss (1948) and McManus (1948) (Pennington, 1949; Bunting, Robinow & Bunting, 1949; Lankford, Hoyo & Lutteringer, 1951). Capsules and slime are demonstrable microscopically by a variety of methods, most satisfactorily in wet films with India ink (Duguid, 1951).

Previous investigations of the influence of cultural conditions on polysaccharide production by bacteria mostly have been made by methods which reveal only part of the polysaccharide. Birch-Hirschfeld (1936) extracted a polysaccharide-containing material from nutrient agar cultures of *Salmonella paratyphi* B, and found greater amounts after growth at 25° than at 37°. Morgan & Beckwith (1939) observed the degree of mucoidness of surface cultures as indicative of the amount of capsule and slime; they found that a low temperature of incubation (10–20°) and the provision of readily utilizable carbohydrate were necessary for highly mucoid growth by salmonella and escherichia strains. Hoogerheide (1939) observed the viscosity of cultures in relation to their cell count and regarded this as a measure of capsule size. He found that the capsules of *Klebsiella pneumoniae* were largest in cultures at 37° and that their size was determined by the amount of sugar supplied in a 4% peptone medium; the size was greatest with 1% glucose, increasing to a maximum after cessation of logarithmic growth; with less glucose it diminished latterly. In defined media capsules were largest when growth was limited by deficiency of phosphate, and very small when calcium was absent. Bukantz (1940) and Bukantz, Cooper & Bullowa (1941) estimated the loose-slime polysaccharide (SSS) of type 3 pneumococci by measurement of the precipitate formed on addition of antiserum to the cell-free supernatant of a blood broth culture; most of the total SSS production occurred after cessation of logarithmic growth, but the rate of SSS production per cell was greatest in the early logarithmic phase and diminished progressively thereafter. Duguid (1948) measured microscopically in wet India ink films the capsules of *A. aerogenes* grown on defined media at 37° for 24 hr.; capsules were largest on media having a high sugar:nitrogen or sugar:phosphorus ratio, wherein growth was limited by deficiency of the nitrogen or phosphorus source so that an excess of sugar, not consumed for growth, remained available for synthesis of capsular polysaccharide. Bunting *et al.* (1949) confirmed these findings for *A. aerogenes* and found that *Chromobacterium prodigiosum* behaved similarly; by periodate-Schiff staining they found that the cells having large capsules also had an unusually large intracellular polysaccharide content. Dagley & Dawes (1949) estimated chemically the polysaccharide in centrifuged deposits of *Bacterium coli* cultures grown in defined media at 38°; the amount of polysaccharide at the end of growth varied with the sugar content of the medium; it diminished during the stationary phase regardless of whether nitrogen- or sugar-exhaustion had halted growth.

METHODS

Surface cultures of *A. aerogenes* were prepared on agar media and measurements were made of their total polysaccharide content, and of the intracellular, capsular and slime fractions separately. The average capsule size and the degree of intracellular polysaccharide staining were observed microscopically. Chemical estimations were made on the deposits of centrifuged cultures for the intracellular and capsular polysaccharides combined, and on the supernatants for the slime polysaccharide. The amount of growth, i.e. of bacterial protoplasm formed, was assessed by a cell count, by an opacity measurement and by a non-dialysable nitrogen estimation. In addition to a visual assessment of the degree of mucoidness, a quantitative expression was obtained indicating the volume of the surface culture in relation to the volume of bacterial protoplasm within it; this was measured as the ratio of the wet weight of the culture to its nitrogen content.

Strains. The recorded observations were made with a single typical strain (A 3) of *Aerobacter aerogenes*. Parallel tests made with other strains of the species showed that these behaved similarly. Strain A 3 corresponded to *Bact. aerogenes* type 1 of Wilson (1935). It had the following properties: non-motile; methyl-red negative; Voges-Proskauer positive; utilized citrate;

indole negative; Eijkman negative; did not liquefy gelatine; fermented glucose, lactose, sucrose, mannitol, glycerol and inositol, but not dulcitol, producing acid and gas. It gave mucoid colonies on sugar-containing media such as MacConkey's agar, at 35–37° and at 15–20°, and formed capsules on all cells together with much loose slime. It had been isolated from human faeces five years previously and thereafter maintained on nutrient agar.

Culture methods. Each surface culture was made on a disk of dialysis cellophan covering 50 ml. agar medium in a Petri dish of 9 cm. diameter. The cellophan enabled rapid and complete removal of the culture for measurement of its wet weight and other estimations. The nutrient substances, lactose, ammonium sulphate and a phosphate mixture were added in various amounts to an agar base which contained per 100 ml. distilled water: washed agar powder, 2 g.; NaCl, 0·2 g.; K_2SO_4, 0·1 g.; $MgSO_4.7H_2O$, 0·02 g.; $CaCl_2.6H_2O$, 0·002 g.; $FeSO_4$, 0·0001 g. For tests of the influence of sulphate content, chlorides were substituted for the sulphates of ammonium, potassium, magnesium and iron, and the required amount of sodium sulphate added. Phosphate was used as a mixture at pH 7·3 of 3 parts by weight of Na_2HPO_4 and 1 part of KH_2PO_4; the amount of the phosphate supply is stated as the combined weight of the two salts. Most media contained 500 mg. phosphate/50 ml.; this proved adequate as a buffer against any marked decrease in pH value resulting from fermentation. In tests of the influence of phosphate deficiency, where the phosphate content was 20 mg. or less/50 ml., it was necessary to provide another buffer; 0·2 or 0·3 % (w/v) sodium bicarbonate was incorporated in the medium and 25 % (v/v) carbon dioxide added to the air of the tank in which the culture was incubated; this $NaHCO_3:CO_2$ system was calculated to buffer at pH 7·2–7·4. The agar base contained small amounts of 'contamination' nitrogen, phosphorus and sulphur compounds; the amount of these was indicated by the amount of growth obtained on medium with none of the corresponding nutrient added.

Disks of cellophan, 10 cm. diameter (dry), were washed and sterilized by boiling in distilled water. With sterile forceps the moist disks were laid on the solidified agar plates so as to cover the whole surface of the agar and turn up at the dish edge. The inoculum was made from an overnight 35° culture in 0·05 % peptone water. A small loopful of this culture, containing about 300,000 cells, was spread over the whole upper surface of the cellophan so as to give a confluent film of growth on it. The plate was incubated with its lid ajar to allow free exchange of air. Thus open, each plate was incubated separately in a sealed glass tank of 8 l. capacity. The 8 l. air available to the 50 ml. plate was calculated to supply sufficient oxygen for complete aerobic utilization of the usual 250 mg. lactose content. An 18 l. tank was used for each of the few cultures made with 1000 mg. lactose/50 ml. Incubation was either for 4 days at 35° or for 14 days at room temperature (15–20°); preliminary tests had shown that these incubations were sufficient to allow virtual completion of growth and polysaccharide production.

Wet weight measurement. Immediately on removal of the plate from the tank after incubation, the cellophan disk together with the whole bacterial growth (moist but without free water) was stripped from the agar, folded up and placed in a weighing bottle; this was at once stoppered and weighed. The wet weight of the culture itself was obtained by subtracting the weight of the empty bottle plus 460 mg., which was the average weight of moist disks without cultures.

Degree of mucoidness. The appearance of the culture was noted and its degree of mucoidness recorded (–, +, + +, + + +) according to how smooth and shiny the surface and how great the transparency in relation its depth on the cellophan.

Final pH *value of medium.* After removal of the disk carrying the culture, 5 ml. distilled water was poured on to the used agar medium and left for 1 hr. to allow diffusion of solutes from agar to water. The water was then decanted and its pH value determined by means of indicators.

Preparation of culture suspension. For the remaining estimations the culture was suspended in a buffered solution (Na_2HPO_4, 0·0375 g.; KH_2PO_4, 0·0125 g.; NaCl, 0·2 g.; K_2SO_4, 0·1 g.; $MgSO_4.7H_2O$, 0·02 g.; distilled water, 100 ml.; pH 7·3). The disk with the culture was rubbed into successive small amounts of solution on the bottom of a large beaker until the whole culture was uniformly suspended in 50 ml.; much rubbing was needed for the highly mucoid cultures.

Average capsule diameter. Capsules were observed in wet films of culture suspension mixed with India ink (Duguid, 1951). The transverse capsule diameter, including the width of the bacillus and the capsular layers on both sides, was measured with an eyepiece micrometer. The average was taken of measurements of the first 50–100 bacilli found in selected parts of the film. From repeated observations it seemed that the error of estimation was less than 10 % when the capsules were large and less than 20 % when small. The width of the bacillus itself was about 0·8 μ. and this should be subtracted from the recorded 'capsule diameter' to obtain the width of the capsular substance alone. The capsules were measured while suspended in a watery film and were thus somewhat expanded above their original size when in the less hydrated conditions of the surface culture (the expansion is seen microscopically when India ink is first added to solid culture material between slide and coverglass). The *average bacillus length*, disregarding the capsular layer, was estimated similarly.

Cell count. A portion of the culture suspension was killed with formalin and an appropriate dilution made. A loopful was placed on a Thoma type counting chamber of 0·02 mm. depth and the bacilli counted by the $\frac{1}{6}$ in. dry objective of a phase contrast microscope. Four chamber preparations were counted for each culture and from the average count was calculated the total number of cells present in the whole culture (i.e. from 50 ml. culture medium).

Intracellular polysaccharide staining. Thick smears were fixed by flaming and stained by the periodate-Schiff method of Hotchkiss (1948), using the non-ethanolic periodic acid 'B' solution and omitting the counterstain. The intensity of red-staining of the bacterial cytoplasm was observed microscopically, using a green filter, and was recorded on a scale ranging from the faintest, almost invisible staining (±), through increasing intensities to a very dark red (+ to ++++).

Opacity measurement. The opacity of the culture suspension was measured by the 'Spekker' photoelectric absorptiometer. The suspension was diluted when necessary to give a drum reading below 0·40, since below this value the relationship between number of bacteria and opacity reading is linear. The reading was multiplied by the dilution factor to express the opacity of the original culture suspension (i.e. culture from 50 ml. medium suspended in 50 ml. saline).

Preparation of culture fractions for chemical estimations. The major part of the culture suspension was centrifuged at 4000 r.p.m. for 30 min. in an angle centrifuge. The deposit of bacterial cells and capsules was washed twice by resuspension in small amounts of saline and further centrifugation, and finally was suspended in 50 ml. fresh saline. The supernatants, which contained the slime, were combined and their volume measured. Before estimation of the polysaccharide, all monosaccharides and oligosaccharides were eliminated by dialysis (which also removed the dialysable nitrogenous substances such as ammonium salts and amino-acids). The culture deposits and supernatants were placed separately in bags of dialysis tubing. These were held in boiling water for 5 min. to inactivate enzymes. The bags were then dialysed against distilled water for 40 hr. The volumes of the dialysed solutions were measured to ensure that they remained unchanged.

Chemical estimation of polysaccharide. Previous estimations of polysaccharide in bacteria have been made by methods depending on a lengthy hydrolysis followed by measurement of reducing value (e.g. Dagley & Dawes, 1949). Recently, in the examination of yeasts, Trevelyan & Harrison (1952) made use of the anthrone method which is more specific for polysaccharide and obviates the need for preliminary hydrolysis. The anthrone method, modified from Seifter, Dayton, Novic & Muntwyler (1950), was used for the present study. We obtained greater reproducibility of results by adding the anthrone reagent slowly instead of rapidly; ten 1 ml. additions were made at 20 sec. intervals to 5 ml. amounts of test solution in tubes of similar size, these being shaken in a cold water bath. Preliminary hydrolysis was not used, since estimations made after hydrolysis with 2 N-sulphuric acid at 100° for periods up to 24 hr. did not give significantly increased values. A comparison was made between this anthrone method and the method of hydrolysis for 20 hr. in 2 N-sulphuric acid at 100° followed by measurement of reducing value by the method of Somogyi (1945). Parallel estimations were made on non-mucoid and highly mucoid cultures grown respectively on medium containing 500 mg. peptone/50 ml. and medium containing 25 mg. peptone and 250 mg. lactose/50 ml. The two methods gave similar polysaccharide values for the non-mucoid culture (polysaccharide:nitrogen ratios 1·04 and 1·1), but the anthrone method gave much higher values than the hydrolysis-Somogyi method for the mucoid culture (polysaccharide:nitrogen ratios 38 and 25 respectively). Although thus giving the higher values, the anthrone method still underestimates somewhat the true amount of polysaccharide; for instance, it does not reveal the uronic acid component. Estimations made in collaboration with Mr W. F. Dudman on extracted and purified polysaccharide from the strain A 3 of *A. aerogenes* suggested that the anthrone method reveals only about 65 % of the total polysaccharide content. Nevertheless, the results of the estimations are recorded in terms of the anthrone value, as mg. glucose equivalent/50 ml. culture.

Estimation of total non-dialysable nitrogen. The non-dialysable nitrogen content of the culture was determined by the micro-Kjeldahl method and recorded as mg. N/50 ml. culture.

Dry weight estimation. A sample of the uncentrifuged culture suspension after dialysis was placed on a watch-glass and weighed after heating in an oven at 130° for 3 hr. The result was recorded as mg./50 ml. culture.

RESULTS

Influence of the nitrogen : sugar ratio in medium

Cultures were grown aerobically for 4 days at 35° on plates of 50 ml. agar medium containing 250 mg. lactose, 500 mg. of the phosphate mixture and different amounts of ammonium sulphate as sole added nitrogen source (the agar also contained contamination nitrogenous compounds equivalent in nutritional value to about 0·5 mg. ammonium sulphate/plate). The results of a typical experiment are given in Table 1 (*a*).

The media with the large contents of ammonium sulphate (250 and 75 mg./50 ml.) gave the maximum amount of growth supportable by their 250 mg. lactose content; utilization of the lactose apparently was complete and directed predominantly towards provision of energy for growth. This was confirmed by the results for cultures grown on a medium with 75 mg. ammonium sulphate and only 50 mg. lactose/50 ml.; these cultures showed proportionate decreases in cell count and nitrogen content to about a fifth. Polysaccharide production was very small on the ammonia-rich media; the cultures appeared non-mucoid, their polysaccharide:nitrogen ratio, wet weight:nitrogen ratio and average capsule diameter were minimal and the cells showed very little intracellular polysaccharide by periodate-Schiff staining. The culture on 75 mg. ammonium sulphate and 250 mg. lactose/50 ml. medium was near the lower limit of polysaccharide production, since the polysaccharide:nitrogen ratio (1·8) was little further diminished by increase of ammonium sulphate to 250 mg. or by decrease of lactose to 50 mg. (Table 1 (*a*)). This minimal polysaccharide production seemingly cannot be dispensed with in favour of greater provision of growth energy, however inadequate the supply of sugar.

The media containing 250 mg. lactose/50 ml. with the smaller contents of ammonium sulphate (25, 7·5, 2·5 and 0·75 mg.) gave proportionately smaller amounts of growth as indicated by the cell count, opacity measurement and nitrogen content. The nitrogen contents of these cultures were nearly equal to the whole amount of the nitrogen available in the medium; this confirms that their growth was limited by exhaustion of the nitrogen supply. Such greatly decreased growths could not have required more than a small fraction

Table 1. *Polysaccharide production by* A. aerogenes *cultures on 50 ml. amounts of medium with different contents of lactose, ammonium salt, phosphate and sulphate*

Medium content (mg.)*			Capsule diameter (μ.)	Intracellular§ polysaccharide stain	Cell count × 10⁻⁹	Opacity	Wet weight (mg.)	Total nitrogen (mg.)	Polysaccharide (mg.)			Wet weight mg./mg. nitrogen	Poly-saccharide mg./mg. nitrogen
Lactose	Ammonium sulphate	Phos-phate‡							Total	Deposit	Super-natant		
(*a*) At 35° for 4 days, aerobically: final pH after growth 6·5–7·1: average cell length 1·3–1·6 μ.													
250	250	500	1·4	..	500	3·38	750‖	8·6	13·1	..	..	87	1·5
250	75	500	1·6	⊥	570	3·32	950	8·2	15·1	12·4	2·7	120	1·8
250	25	500	2·5	..	190	2·13	1470‖	4·2	28·9	..	..	350	6·9
250	7·5	500	4·3	++	52	1·05	2910	1·6	46·1	24·8	21·3	1800	29
250	2·5	500	4·2	..	20	0·45	1000‖	0·63	18·0	..	..	1600	29
250	0·75	500	3·9	..	7·2	0·20	370	0·28	9·0	5·9	3·1	1300	32
250	0†	500	3·7	..	2·3	0·07	50	0·11	2·8	..	..	450	25
50	75	500	1·5	..	110	0·78	170	2·0	2·2	2·0	0·2	85	1·1
1000	7·5	500	3·7	..	46	1·12	2900	1·6	48·1	25·5	22·6	1800	30
(*b*) At 15–20° for 14 days, aerobically: final pH 6·6–6·9: average cell length 1·5–1·7 μ.													
250	75	500	1·9	+	350	4·48	1100	8·3	25·8	23·8	2·0	130	3·1
250	7·5	500	4·7	++++	38	1·26	1800	1·6	47·3	31·4	15·9	1100	30
(*c*) Anaerobically, at 35° for 4 days: final pH 6·5–6·7: average cell length 1·3–1·5 μ.													
250	75	500	1·4	⊥	120	0·98	350	2·0	1·7	1·0	0·7	180	0·86
250	7·5	500	1·3	⊥	36	0·47	160	1·1	2·8	1·7	1·1	150	2·5
250	2·5	500	1·5	⊥	17	0·20	50	0·48	1·3	0·74	0·56	100	2·7
(*d*) Inadequately buffered, at 35° for 4 days, aerobically: final pH 4·1–4·2: average cell length 5·2–6·3 μ.													
250	75	7·5	2·0	+	3·8	0·41	<30	0·60	1·05	0·62	0·43	<50	1·7
250	7·5	7·5	1·9	+	5·3	0·50	<30	0·68	0·77	0·55	0·22	<44	1·1
(*e*) At 35° for 4 days, aerobically, 100 mg. sodium bicarbonate buffer: final pH 6·5–7·3: average cell length 1·2–1·9 μ.													
250	250	6	1·7	⊥	620	3·90	1010	8·9	14·9	10·9	4·0	110	1·7
250	250	0·6	3·7	++	83	1·44	2980	1·71	49·8	25·9	23·9	1700	29
250	250	0·2	4·3	++	26	0·59	1610	0·73	29·0	11·6	17·4	2200	40
(*f*) At 35° for 4 days, aerobically, with 250 mg. lactose, 250 mg. ammonium chloride and 500 mg. phosphate													
Sodium sulphate content	2		1·5	⊥	320	4·27	760	8·8	11·3	8·9	2·4	86	1·3
	0·2		2·9	++	82	1·59	1380	2·4	25·2	15·6	9·6	580	11
	0†		2·9	++	32	0·75	610	0·9	15·7	..	..	680	17

* (*a*)–(*e*) on medium base with excess sulphate; (*f*) sulphate-free medium base.

† 'Contamination' nitrogen and sulphur compounds present.

‡ Mixture of Na_2HPO_4, 3 parts by weight, and KH_2PO_4, 1 part: pH 7·3.

§ Intracellular polysaccharide staining intensity.

‖ Dry weight of cultures in 1st, 3rd and 5th rows of (*a*) was 91, 92 and 45 mg. respectively.

of the available lactose for provision of growth energy, and so must have retained a large excess available for extra polysaccharide synthesis. In fact, polysaccharide production was very great on the ammonia-poor media; the cultures were highly mucoid and their cells showed much intracellular polysaccharide by periodate-Schiff staining (Pl. 1, fig. 5); as compared with the ammonia-rich cultures, their polysaccharide: nitrogen and wet weight: nitrogen ratios were increased up to 20-fold, and their average capsule diameter up to 3-fold. The culture on 7·5 mg. ammonium sulphate and 250 mg. lactose/50 ml. was near to the upper limit of polysaccharide production, since its high polysaccharide: nitrogen ratio (29) was not exceeded significantly when the ammonium sulphate was decreased to 2·5 mg. or less, or when the lactose was increased to 1000 mg. (Table 1 (*a*)). Apparently there is an upper limit beyond which polysaccharide production does not continue, even when an abundance of unused sugar remains available. Production was not halted because of cell death; cell viability after growth for 4 days at 35° was 90–100 % in the ammonia-poor cultures and 70–80 % in the ammonia-rich cultures.

Compared with the non-mucoid culture on 75 mg. ammonium sulphate and 250 mg. lactose/50 ml., the mucoid culture on 7·5 mg. ammonium sulphate and 250 mg. lactose/50 ml. gave only a fifth the amount of growth, as measured by the nitrogen content. Thus if the latter culture had produced relatively no more polysaccharide than the former (i.e. 3 mg., a fifth of 15·1 mg.), it would have utilized for growth and polysaccharide synthesis only a fifth of the available 250 mg. lactose; about 200 mg. lactose was therefore available for production of the extra polysaccharide in the mucoid culture, namely 43 mg. in anthrone value. Since the actual polysaccharide content is about 1½ times the anthrone value, these results suggest that about 60 mg. polysaccharide was synthesized from 200 mg. lactose. The polysaccharide content, similarly corrected (anthrone value × 1½), composed about 20 % of the dry weight of the non-mucoid culture on 250 mg. ammonium sulphate and about 60 % of the dry weight of the mucoid culture on 2·5 mg. ammonium sulphate/50 ml. (Table 1 (*a*)).

The cultures grown with little ammonium sulphate (7·5 mg. or less/50 ml.) had twice as great a nitrogen content per cell, and thrice as great an opacity measurement per cell as the cultures made with much ammonium sulphate (250 or 75 mg./50 ml.). This was due in part to difference in cell size; measurements made on photomicrographs of wet films indicated that the average cell volume (i.e. of protoplasm, but not capsule) was 1½–2-fold greater in the ammonia-poor cultures. The greater nitrogen content per cell in the ammonia-poor cultures was also due in part to the nitrogenous component of their more abundant capsules and slime.

In experiments made with peptone (Bactopeptone) as nitrogen source the results were similar to those obtained with ammonium sulphate. Cytological studies were made of cultures on 250 mg. lactose and 25 mg. peptone/50 ml. medium, which had large capsules and much slime. By the periodate-Schiff method, strain A 3 and most other *A. aerogenes* strains gave strong staining of their intracellular polysaccharide, but no visible staining of their capsules and slime (cf. Pl. 1, fig. 4); an exceptional strain, A 4, showed staining of the capsular polysaccharide in addition to the intracellular (Pl. 1, fig. 2). Dried films, not shadowcast, were examined by the electron microscope. In most fields the bacillary outlines were obscured by dense shrunken capsular material, but in a few there was good differentiation. Thus Pl. 1, fig. 1 shows dark bacilli surrounded by lighter capsules, and these surrounded by still lighter slime. The capsules and slime appear continuous in substance, but are clearly demarcated by the difference in density. The slime continuum is fenestrated by shrinkage due to drying.

Influence of the phosphate: sugar ratio

Cultures were grown for 4 days at 35° on plates of 50 ml. bicarbonate-buffered medium containing 250 mg. lactose, 250 mg. ammonium sulphate and different amounts of phosphate (Na_2HPO_4, 3 parts by weight; KH_2PO_4, 1 part) as sole added phosphorus source (the agar also contained contamination phosphorus compounds equivalent in nutritional value to about 0·1 mg. phosphate/50 ml.). The results for some typical cultures are shown in Table 1 (*e*). The effect of phosphate limitation was similar to the effect of nitrogen limitation. The culture on 6 mg. phosphate/50 ml. medium gave maximal growth and minimal polysaccharide production; increase of phosphate supply to 20 mg./50 ml. did not further increase the amount of growth nor decrease the polysaccharide. The culture on 0·2 mg. phosphate/50 ml. gave a much smaller amount of growth and nearly maximal polysaccharide production, as indicated by a polysaccharide: nitrogen ratio of 40. Further decrease of the phosphate supply did not give greater production of polysaccharide; e.g. a polysaccharide: nitrogen ratio of 36 was obtained in a lesser growth (0·20 mg. nitrogen content) given by a medium without added phosphate. Moreover, relatively little further increase of polysaccharide production resulted from a 4-fold increase of the lactose supply; a culture on 1000 mg. lactose and 0·2 mg. phosphate/50 ml. medium showed a polysaccharide: nitrogen ratio of 48. The maximal polysaccharide production on phosphate-poor media was about 50% greater than the maximal on nitrogen-poor media. This difference was not due to the use of bicarbonate buffer in the phosphate-poor media; the nitrogen-poor media did not produce increased amounts of polysaccharide when buffered with bicarbonate and supplied with only 6 mg. phosphate/50 ml. instead of 500 mg./50 ml.

Influence of the sulphate: sugar ratio

Cultures were grown for 4 days at 35° on plates of 50 ml. medium containing 250 mg. lactose, 250 mg. ammonium chloride, 500 mg. phosphate mixture and different amounts of sodium sulphate as sole added sulphur source (the agar also contained contamination sulphur compounds equivalent in nutritional value to about 0·1 mg. sodium sulphate/50 ml.). The results for three representative cultures are given in Table 1 (*f*). Sulphate limitation was found to have a similar effect to nitrogen and phosphate limitations. Maximal growth and minimal polysaccharide production were obtained on the medium with 2 mg. sodium sulphate/50 ml.; a medium with the sulphate increased to 6 mg./50 ml. gave the same amount of growth and polysaccharide. Maximal polysaccharide production was obtained in the greatly decreased growth on medium without added sodium sulphate (i.e with 'contamination' sulphur compounds only). No further increase of polysaccharide was obtained by 4-fold increase of the lactose supply in this medium to 1000 mg./50 ml. The maximal polysaccharide production on the sulphate-poor media, as measured by the polysaccharide:nitrogen ratio (e.g. 17), was only about half the maximal on nitrogen-poor media.

Influence of low temperature of incubation

Cultures were grown for 14 days at room temperature (15–20°) on the phosphate-buffered lactose media containing different amounts of ammonium sulphate. The results for two representative cultures are shown in Table 1 (*b*), and may be compared with the results for the corresponding cultures at 35° shown in Table 1 (*a*). The results for the two temperatures of incubation were generally similar. On a given medium the amount of growth as measured by the nitrogen content was the same at both temperatures. The 15–20° cultures contained a smaller number of cells than the 35° cultures, and these were slightly larger in size. The maximal polysaccharide production on the ammonia-poor media was the same at both temperatures, but the minimal production on the ammonia-rich media was almost twice as great at 15–20° as at 35°. Thus, it is only in conditions of limited sugar and abundant nitrogen supply that a low temperature of incubation favours increase of mucoidness and polysaccharide production in *A. aerogenes*. The extent of such increase appears small compared with that observed by Birch-Hirschfeld (1936) and Morgan & Beckwith (1939) for salmonella and escherichia strains. The 15–20° cultures on the ammonia-rich media gave higher opacity and dry-weight measurements than the corresponding 35° cultures (e.g. dry weight 106 mg. as compared with 91 mg.); probably this was due to their higher polysaccharide content. The 15–20° cultures yielded a greater proportion of their polysaccharide in the centrifuged cellular deposit and a smaller proportion in the supernatant; this was correlated with their slightly larger capsule size and their much greater intracellular polysaccharide content as shown by periodate-Schiff staining (cf. Pl. 1, figs. 5 and 6).

Influence of lack of oxygen

Cultures were grown in an anaerobic jar for 4 days at 35° on the phosphate-buffered lactose media containing different amounts of ammonium sulphate. The results for these cultures grown in the absence of molecular oxygen are shown in Table 1 (*c*), and may be compared with those for the corresponding aerobic cultures shown in Table 1 (*a*). The anaerobic culture on 250 mg. lactose and 75 mg. ammonium sulphate/50 ml. had about the same cell count, nitrogen content and polysaccharide content as the aerobic culture on 50 mg. lactose and 75 mg. ammonium sulphate/50 ml. Since growth on these ammonia-rich media was limited by the energy source, the result suggests that anaerobic utilization of the lactose yielded little more than a fifth of the energy obtainable aerobically.

The anaerobic cultures on the media with 250 mg. lactose and only 7·5 or 2·5 mg. ammonium sulphate/50 ml. gave nearly as much growth as the aerobic cultures on the same media, but they did not give the very greatly increased polysaccharide production of the latter; their polysaccharide: nitrogen ratios little exceeded the minimum obtained in ammonia-rich cultures. The lactose supply in the ammonia-poor media under aerobic conditions constituted an excess providing for much extra polysaccharide synthesis, but under anaerobic conditions sufficed only for growth and a small polysaccharide production. It is possible that anaerobiosis is specifically unfavourable to polysaccharide synthesis, apart from its effect in decreasing the amount of energy obtainable from the sugar supply. This is suggested by the finding that the polysaccharide: nitrogen ratio was very little greater in the anaerobic culture on 2·5 mg. ammonium sulphate/50 ml. than in that on 7·5 mg./50 ml., in spite of there being available to the former more than twice as much sugar relative to the amount of growth.

Influence of degree of acidity

Cultures were grown for 4 days at 35° on plates of 50 ml. medium containing 250 mg. lactose, large (75 mg.) or small (7·5 mg.) amounts of ammonium sulphate, and an amount of phosphate (7·5 mg.) which was sufficient nutritionally for maximal growth, but quite inadequate as a buffer against development of an acid reaction from fermentation. The results are shown in Table 1 (*d*), and may be compared with those for the corresponding well-buffered cultures in Table 1 (*a*). The poorly buffered cultures became much more acid (e.g. pH 4·1) than the well-buffered cultures, and their growth was considerably less in amount. The bacilli composing the acid cultures were exceptionally large, with average length about four times that of the cells from the well-buffered cultures, and this was correlated with a larger nitrogen content per cell; they also differed from the normal bacilli in containing numerous granules of volutin.

The limitation of growth by acidity did not result in an increased production of polysaccharide; the polysaccharide: nitrogen ratio, wet weight: nitrogen ratio and capsule size were minimal. Furthermore, polysaccharide production was no greater in the acid culture on the ammonia-poor medium than in that on the ammonia-rich medium. Apparently an excess of energy source remained unutilized. It was therefore concluded that an acid reaction is inhibitory to polysaccharide synthesis, even at a pH value not low enough to prevent entirely the continuance of protoplasmic growth. If polysaccharide is required for septum formation, and thus for cell division, this might explain the abnormally long cells in acid cultures.

Course of polysaccharide production during growth

A series of cultures were grown on plates of 50 ml. nitrogen-poor medium containing 250 mg. lactose, 8·3 mg. ammonium sulphate and 500 mg. phosphate mixture, and were incubated at 35° for different periods ranging from 8 to 168 hr. The results for these separate cultures of different age are shown in Fig. 1. The phase of logarithmic growth extended approximately from 5 to 14 hr. (cell count 9·6 × 10^9 and nitrogen content 0·82 mg. at 14 hr.); thereafter growth continued at a decreasing rate until 24 hr., when the cell count (42 × 10^9) and nitrogen content (1·7 mg.) were nearly maximal. Polysaccharide production occurred throughout the first 3–4 days of incubation, but the greater part of the polysaccharide (about 95 % of the 44 mg. total) was produced after the end of the logarithmic phase; the main increase in the polysaccharide:nitrogen ratio (from 2·3 to 23) took place between 14 and 48 hr.

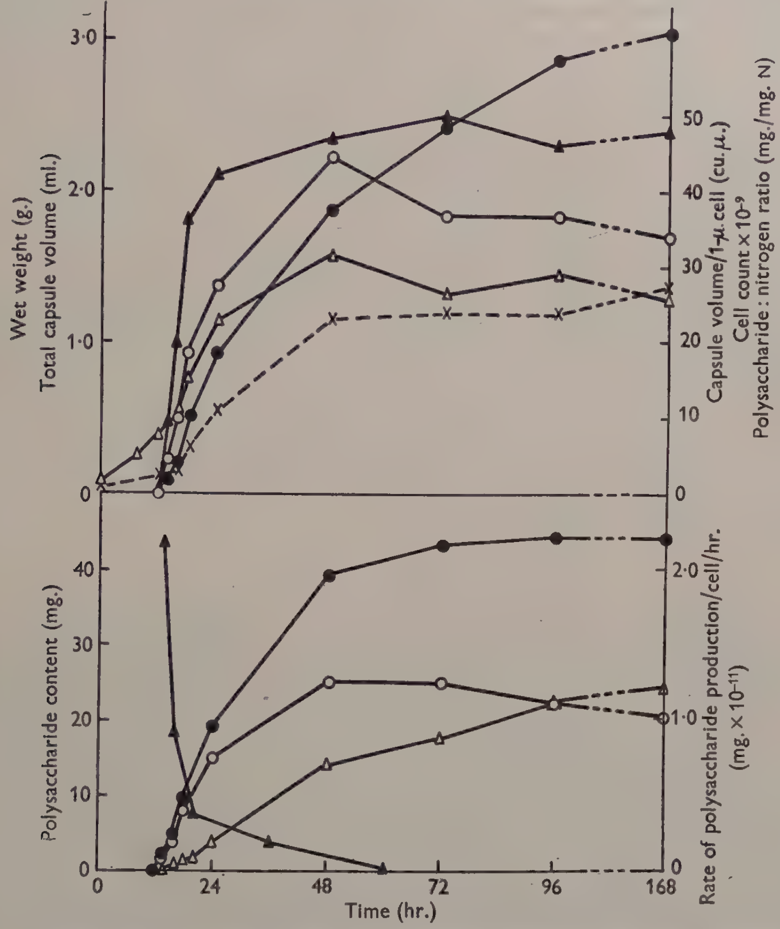


Fig. 1. Polysaccharide production, wet weight, capsule volume and cell count during growth on 50 ml. medium containing 250 mg. lactose and 8·3 mg. ammonium sulphate. Ordinates: (1) *Top left*; wet weight (g.), —●—●—; total capsule volume in suspension (ml.), —○—○—. (2) *Top right*: capsule volume/1-μ. cell (cu. μ.), —Δ—Δ—; cell count $\times 10^{-9}$, —▲—▲—; polysaccharide : nitrogen ratio (mg./mg. N), —×- - -×—. (3) *Bottom left*: total polysaccharide content (mg.), —●—●—: deposit polysaccharide content (mg.), —○—○—; supernatant polysaccharide content (mg.), —Δ—Δ—. (4) *Bottom right*, rate of polysaccharide production/cell/hr. (mg. $\times 10^{-11}$), —▲—▲—.

However, the rate of polysaccharide production per cell was actually greatest during the logarithmic phase, and declined progressively thereafter. Fig. 1 shows this variation in the rate of total polysaccharide production/cell/hr. by *A. aerogenes* calculated according to the formulae used by Bukantz *et al.* (1941) for the rate of SSS production by pneumococci. The separate estimations for the culture deposits and supernatants showed that the main production of slime polysaccharide occurred later than the main production of intracellular and capsular polysaccharides; after 24 hr. the slime accounted for only *c.* 20% of the total polysaccharide, while after 96 hr. it accounted for *c.* 50%.

On first becoming clearly visible after 12 hr. growth, the culture appeared non-mucoid, but acquired a highly mucoid character after 24 hr. The wet weight:nitrogen ratio increased throughout the 7 days to reach 1900, but the main increase (from 110 to 1100) took place between 14 and 48 hr., concurrent with the main increase of polysaccharide. Intracellular polysaccharide staining was faint and uniform in the logarithmic phase (e.g. ⊥, Pl. 1, fig. 3), increased to a maximal intensity at 24–48 hr. (e.g. + + +, Pl. 1, fig. 4) and then diminished, becoming faint in an increasing proportion of cells (e.g. + +, at 96 hr., Pl. 1, fig. 5). The average capsule diameter similarly increased during and after the logarithmic phase from the original size of 1·6 μ. to a maximum of 4·3 μ. at 48 hr.; subsequently it diminished very slightly (e.g. 3·9 μ. at 168 hr.). The average volume of capsular material in cu.μ. per standardized cell of 1 μ. length was calculated approximately as:

$$\frac{1}{L}\left[\frac{4}{3}\pi R^3+\pi R^2(L-1)-\pi r^2 L\right],$$

where R = half the 'average capsule diameter', r = bacillus radius (taken as 0·4 μ. throughout), and L = average bacillus length (e.g. 3·5 μ. at 8 hr. and 1·5 μ. at 18 hr.). The increase during growth of this average capsule volume/1-μ. cell is shown in Fig. 1. Multiplication of this volume by the total cell count and average bacillus length gave the total capsule volume of the culture suspension. This latter provided the only measure obtainable of the total capsular polysaccharide exclusive of intracellular polysaccharide, and its increase during growth is shown in Fig. 1. In the first 48 hr. the calculated total capsule volume of the culture suspension was greater than the volume of the whole surface culture before suspension, measured by the wet weight converted to ml.; presumably this was due to the capsular expansion which occurs on suspension in water. After 48 hr. the surface culture volume became the greater, presumably because of its increasing content of slime.

DISCUSSION

The present chemical estimations confirm and illustrate quantitatively the finding of Duguid (1948) that deficiency of nitrogen or phosphate greatly increases polysaccharide production by *A. aerogenes*. They show also that deficiency of sulphate acts likewise. This gives further support to the hypothesis that deficiency of an essential nutrient other than the carbohydrate source of energy, by limiting growth and thus the energy requirement for growth, conserves carbohydrate for synthesis of polysaccharide. Also in agreement was the finding that polysaccharide production was not increased when limitation of growth was achieved by decrease in the sugar supply or by exclusion of oxygen, the latter decreasing the amount of available energy from the given sugar supply. An upper limit was found to the amount of polysaccharide formed by cultures deficient in nitrogen-source, phosphate or sulphate; production was ended while the sugar and oxygen were unexhausted, the pH value favourable and most of the bacilli still alive. The factors responsible for this limitation are not known.

With varying conditions, the intracellular, capsular and slime polysaccharides generally increased or decreased together, though not in strict proportion. Thus, of the total polysaccharide content after 4 days at 35°, the slime comprised 35–63 % in the highly mucoid cultures, but only 9–27 % in the non-mucoid cultures. The proportion of slime polysaccharide in the 15–20° cultures was only about half that in the 35° cultures. The wet weight:nitrogen ratio proved a satisfactory measure of the degree of mucoidness; under all conditions it varied in concordance with the degree of mucoidness as judged visually, and also in close proportion with the polysaccharide:nitrogen ratio.

The best measure of the amount of protoplasmic growth proved to be the non-dialysable nitrogen content. Only a minor amount of non-dialysable nitrogenous matter seemed to occur extracellularly in the capsules and slime; between 1 and 20 % of the total nitrogen was found in the supernatants, and another similar proportion may have been present in the capsules, though no separate measurement could be obtained of this. Thus it seems that little error is entailed in regarding the nitrogen content as a measure of the intracellular proteinous matter. The opacity and dry weight measurements were influenced too greatly by the polysaccharide content of the culture to serve as satisfactory measures of the amount of protoplasm. The cell count was misleading when the cell size was abnormal, as in the acid cultures.

It is generally recognized that in regard to the cytology, chemical constitution and metabolic activity of bacteria, a distinction must be made between the 'young' cells of the early logarithmic phase and the 'old' cells of the stationary phase. The present observations, with those made previously by staining for volutin and nuclear bodies (Duguid, 1948), indicate that in *A. aerogenes* a further distinction must be made of different types among the stationary-phase cells, these types differing according to the conditions which limit growth and initiate the stationary phase. First, the cells from cultures limited by exhaustion of the carbon and energy source are of the type generally considered 'normal', having normal length, small capsules and a low polysaccharide content. Secondly, the cells from cultures limited by exhaustion of the nitrogen-source, phosphate or sulphate are distinguished by their much larger capsules and polysaccharide content, and by the ease with which their nuclear bodies are revealed by simple staining; furthermore, the phosphate-starved cells are distinguished from the others by their habit of forming intracellular granules of volutin during the first hours of subcultivation on fresh medium containing phosphate. Thirdly, the cells from cultures limited by developing acidity are distinguished by their abnormally great length, their small capsules and polysaccharide content, and by their possession of numerous volutin granules.

The authors wish to express their thanks to Professor T. J. Mackie for his interest and encouragement.

REFERENCES

Birch-Hirschfeld, L. (1936). Zur Analyse der hydrophilen Kolloide in den Kolonien von Paratyphus B. *Z. Hyg. InfektKr.* **117**, 626.

Bukantz, S. C. (1940). Relation between growth of Pneumococcus III and concentration of capsular polysaccharide appearing in culture filtrates. *Proc. Soc. exp. Biol., N.Y.* **43**, 553.

Bukantz, S. C., Cooper, A. & Bullowa, J. G. M. (1941). The elaboration of soluble capsular polysaccharide by Pneumococcus III in relation to growth phases *in vitro*. *J. Bact.* **42**, 29.

Bunting, M. I., Robinow, C. F. & Bunting, H. (1949). Factors affecting the elaboration of pigment and polysaccharide by *Serratia marcescens*. *J. Bact.* **58**, 114.

Dagley, S. & Dawes, E. A. (1949). Factors influencing the polysaccharide content of *Escherichia coli*. *Biochem. J.* **45**, 331.

Duguid, J. P. (1948). The influence of cultural conditions on the morphology of *Bacterium aerogenes* with reference to nuclear bodies and capsule size. *J. Path. Bact.* **60**, 265.

Duguid, J. P. (1951). The demonstration of bacterial capsules and slime. *J. Path. Bact.* **63**, 673.

Hoogerheide, J. C. (1939). Studies on capsule formation. I. The conditions under which *Klebsiella pneumoniae* (Friedländer's bacterium) forms capsules. *J. Bact.* **38**, 367.

Hotchkiss, R. D. (1948). A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. *Arch. Biochem.* **16**, 131.

Lankford, C. E., Hoyo, H. & Lutteringer, J. F. (1951). Intracellular polysaccharide of *Enterobacteriaceae*. *J. Bact.* **62**, 621.

McManus, J. F. A. (1948). Histological and histochemical uses of periodic acid. *Stain Tech.* **23**, 99.

Morgan, H. R. & Beckwith, T. D. (1939). Mucoid dissociation in the colon-typhoid-salmonella group. *J. infect. Dis.* **65**, 113.

Pennington, D. (1949). The use of periodate in microbiological staining. *J. Bact.* **57**, 163.

Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). The estimation of glycogen with the anthrone reagent. *Arch. Biochem.* **25**, 191.

Somogyi, M. (1945). A new reagent for the determination of sugars. *J. biol. Chem.* **160**, 61.

Trevelyan, W. E. & Harrison, J. S. (1952). Studies in yeast metabolism. *Biochem. J.* **50**, 298.

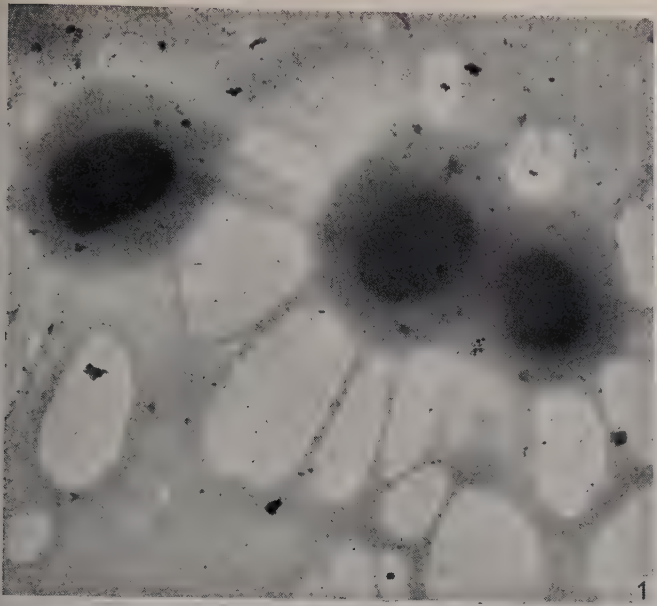
Wilson, G. S. (1935). The bacteriological grading of milk. *Spec. Rep. Ser. med. Res. Coun., Lond.* no. 206, p. 156.

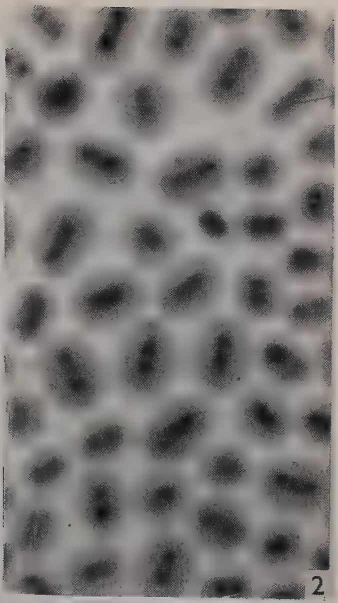
EXPLANATION OF PLATE

Cultures of *A. aerogenes* on phosphate-buffered agar.

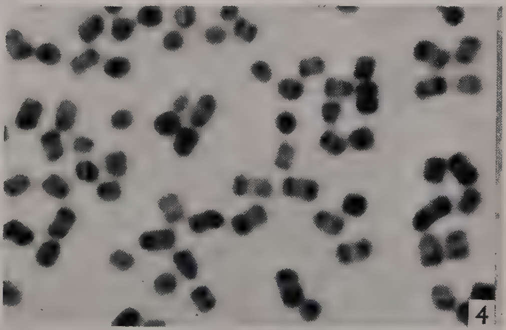
Fig. 1. Strain A 3. After 48 hr. at 35° on lactose 250 mg. and peptone 25 mg./50 ml. Unfixed and unshadowed film by electron microscope at 75 kv. Dark bacilli with lighter capsules surrounded by even lighter slime which is fenestrated by drying. (× 12,000.)

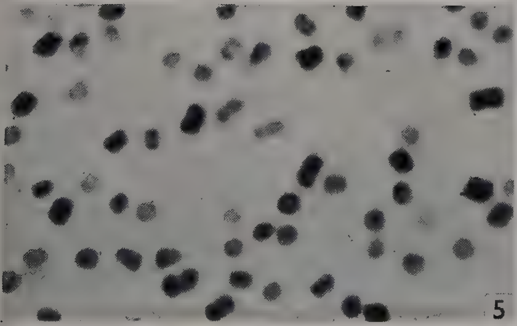
Journal of General Microbiology, Vol. 9, No. 2

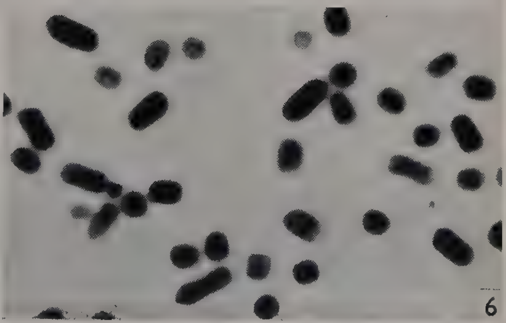

1


2

3


4


5


6

J. P. DUGUID & J. F. WILKINSON—*AEROBACTER AEROGENES* POLYSACCHARIDES. PLATE 1

Fig. 2. Strain A 4. After 24 hr. at 35° on lactose 250 mg. and peptone 25 mg./50 ml. Periodate-Schiff stain for polysaccharide. Intense bipolar staining of cytoplasm and lighter staining of capsules. (×3000.)

Figs. 3–6. Strain A 3. On lactose 250 mg. and ammonium sulphate 8·3 mg./50 ml. Periodate-Schiff stain. (×3000.)

Fig. 3. After 10 hr. at 35°; slight uniform staining (+).

Fig. 4. After 24 hr. at 35°; intense bipolar staining (+ + +).

Fig. 5. After 96 hr. at 35°; intense staining in some cells and slight staining in others (+ +).

Fig. 6. After 14 days at 15–20°; very intense staining (+ + + +).

(*Received* 17 *February* 1953)

Gyllenberg, H., Rossander, M. & Roine, P. (1953). *J. gen. Microbiol.* 9, 190–198.

A Strain of *Lactobacillus bifidus* which requires Strepogenin

By H. GYLLENBERG, MARJATTA ROSSANDER and P. ROINE
Department of Nutritional Chemistry, University of Helsinki, Finland

SUMMARY: A strain of *Lactobacillus bifidus* was isolated from the faeces of a breast-fed infant and subcultivated in the minimal medium of Hassinen, Durbin, Tomarelli & Bernhart (1951). The cysteine requirement for maximal growth of the strain was *c.* 100 μg. cysteine/ml. Human and cow milk were found to contain another factor, besides cysteine, which was essential for optimal growth. The active material in milk could be replaced by enzymic hydrolysates of some commercial proteins; egg albumin, and blood albumin were the most potent sources. The active material was heat stable, but was completely inactivated by treatment with strong acid. All the properties of this active material studied showed a close resemblance to those of strepogenin.

Until recent years some confusion has existed regarding the definition of the term *Lactobacillus bifidus*. This is especially true concerning the relations of the strains investigated to Tissier's (1899, 1900) original *Bacillus bifidus communis*. Many strains with different properties have been claimed as the authentic *L. bifidus* in the sense of Tissier's description. This confusion seems to have been cleared up by the investigations of Norris, Flanders, Tomarelli & György (1950). They found that strains which on isolation exhibited a typical bifid morphology rapidly changed to straight rods of common lactobacillus type when subcultivated in complex organic media. A change took place also in their relation to atmospheric oxygen, the originally anaerobic or micro-aerophilic strains becoming capable of aerobic growth. When, however, subcultivation was carried out in the simplified medium of Teply & Elvehjem (1945) the original properties remained unchanged for practically unlimited periods of time. Since the properties of the unchanged type agree with those of Tissier's *B. bifidus communis*, it should, according to Norris *et al.* (1950), be named *Lactobacillus bifidus*, and the straight rod type, since it is a variety of the unchanged type, *Lactobacillus parabifidus*. This suggested nomenclature is the reverse of that in the current edition of *Bergey's Manual* (1948). The description of *L. bifidus* in *Bergey's Manual* is based on the investigation of Weiss & Rettger (1938). The importance of a carefully controlled isolation and subcultivation technique was evidently overlooked by earlier investigators, a fact which possibly explains the disagreement between their results.

For strains of the straight rod type Tomarelli and his co-workers (1949*a*, 1949*b*, 1950) demonstrated nutritional requirements which closely resemble those of other lactobacilli. For example, deoxyribonucleic acid derivatives as well as unsaturated fatty acids are required by this type of *L. bifidus*. The nutritional requirements of the bifid type seem to be simpler. Hassinen *et al.* (1951) found that in a medium containing cystine (or cysteine) strains of the bifid type utilized ammonium salts as a nitrogen source. Of the B-group vitamins only biotin and pantothenic acid were required; purine and pyrimidine compounds were not essential for the strains studied. Tomarelli *et al.* (1950) found that unsaturated fatty acids were required by the bifid type also, when the medium contained no acetate.

Hassinen *et al.* (1951) found that the bifid type remained unchanged in minimal nutrient media. Accordingly, it seems evident that continuous subcultivation under these conditions prevented change in the nutritional requirements of *L. bifidus*. While investigating a strain of *L. bifidus* isolated and subcultivated under the conditions described by Hassinen *et al.* (1951), the present authors found that their strain required milk as a supplement to the basal medium. The investigations described below were carried out in order to explore this observation.

EXPERIMENTAL

Methods

The strain TM 2 of *L. bifidus* used chiefly was isolated from the faeces of a breast-fed infant by means of the medium of Hassinen *et al.* (1951). In agreement with Hassinen *et al.* (1951) only typical *L. bifidus* colonies grew on this medium. No growth was observed under aerobic conditions and no production of carbon dioxide could be demonstrated. Certain pentoses in addition to the usual hexoses and disaccharides were fermented; the fermentation products were not analysed.

In experiments on nutritional requirements of the isolated strains inoculation of the assay tubes was carried out from saline suspensions of cells. Anaerobic conditions were obtained by pouring melted paraffin into each tube and allowing it to solidify on the liquid surface, thus forming a plug. The tubes were incubated at 37° for 65–70 hr. A measure of growth was obtained by titration of the acid produced; this was not calibrated against increase of cell mass or turbidity.

In investigating the strepogenin activity of protein hydrolysates some experiments with *L. casei* (ATCC 7469) were performed according to Kodicek & Mistry (1952) and with the medium of Clegg, Kodicek & Mistry (1952), but the growth was measured by titration of acid after 48 hr. growth, and not turbidimetrically as proposed by these authors for the strepogenin assay.

The whey used in some experiments was prepared as follows. Milk was curdled with rennet and the coagulum removed by filtration through a cloth. The filtrate was adjusted to *c.* pH 5·2 by addition of dilute acetic acid. This treatment gave a lactalbumin precipitate which was filtered off. This filtrate was readjusted to pH 5·2 with acetic acid and brought to the boil. Additional protein (lactoglobulin) was thereby precipitated and filtered off. The remaining greenish-yellow clear liquid still contained 0·6–0·65 mg. N/ml., and remained clear on further addition of acetic acid.

Cysteine (cystine) requirement

While studying the growth of freshly isolated *L. bifidus* strains in human or cow milk it was observed that growth in human milk was greatly improved when prolonged heating at 63° was used for 'sterilization' instead of autoclaving (Table 1). When cysteine was added to the milk, however, no distinct difference could be found in acid production as between autoclaved milk or milks heated to 63°. Thus it appeared that the strains were dependent on the presence of cysteine in the medium, and that autoclaving made cysteine unavailable. Similar observations were also made when autoclaved milk or milks heated at 63° were added as supplements to the basal medium for *L. bifidus* when prepared cysteine-free (Fig. 1). Here again autoclaved human milk was distinctly less active in replacing cysteine in this medium. Cow milk seemed to contain, even when not autoclaved, only very small amounts of material which could replace cysteine for *L. bifidus*, as compared with human milk. This is noteworthy since cow milk contains about the same total amount of cysteine (cystine) as human milk.

Table 1. *Effect of different heat treatments of human milk on the growth of* L. bifidus (*TM* 2 *and KA* 2)

	Strain TM2		Strain KA2	
	Milk heated 10 min. at 110°	Milk heated 30 min. at 63°	Milk heated 10 min. at 110°	Milk heated 30 min. at 63°
	ml. 0·1N-NaOH/10 ml. milk			
Blank (not inoculated)	2·0	1·0	2·0	1·0
Human milk	7·9	14·2	7·6	14·3
Human milk + cysteine (200 μg./ml.)	19·0	16·8	15·8	16·6

Further experiments showed that addition of cysteine alone to the medium did not produce maximal growth. As can be seen from Table 2, small amounts of milk had a distinct growth-promoting effect independent of the kind of heat treatment or of milk. This effect appeared to be due to an additional essential factor, and the cysteine requirement could be determined only when the basal medium contained a supplement of this factor. The medium was therefore modified by an addition of a small amount of autoclaved cow milk, the cysteine activity of which was negligible (Fig. 1). With this supplemented medium the minimum cysteine requirement of the test strains was *c.* 100 μg. cysteine/ml. medium (Fig. 2).

Requirement for the additional factor present in milk

The additional effect of milk, apart from that due to cysteine, is shown in Table 2. The curves in Figs. 3 and 4 show that there was a direct relationship between the amount of milk and the growth of test organism. It can be seen, also, that the active factor of milk is heat stable: the same effect was obtained whether or not the milk was autoclaved. After partial removal of the protein the active factor was found in the whey.

Experiments were carried out in order to establish the nature of the active substance. The results are tabulated in Table 3. It was found that the growth-promoting activity of milk (and whey) was not due to any vitamin of the B-complex, nucleic acid derivatives, components of the milk ash, or fatty acids (Tween 80). Amino-acid mixtures prepared to simulate the amino-acid composition of milk and some milk fractions were also inactive. The only preparations which showed activity in the first experiments were tryptic digests of casein and lactalbumin separated from milk. Accordingly, similar

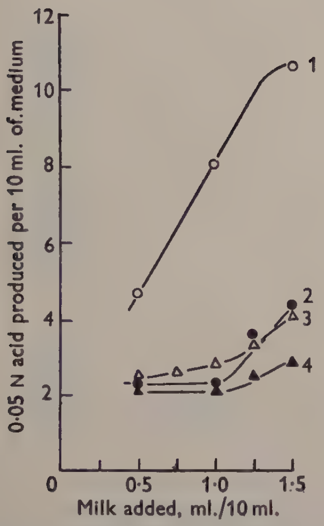


Fig. 1

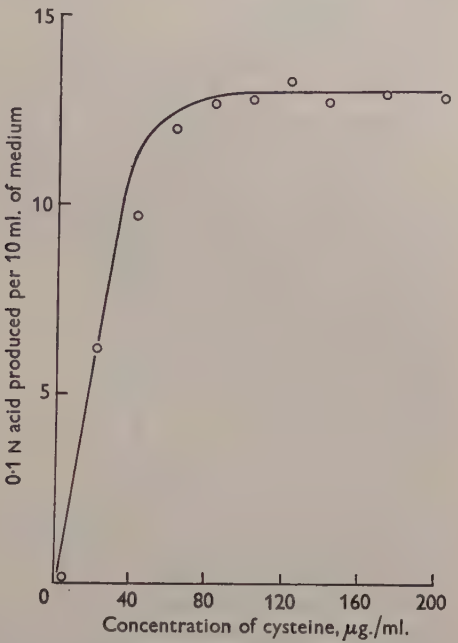


Fig. 2

Fig. 1. Effect of human milk or cow milk on the growth of *L. bifidus* (TM2) in a medium containing no cysteine. 1. Human milk, heated for 30 min. at 63°. 2. Human milk, heated for 10 min. at 110°. 3. Cow milk, heated for 30 min. at 63°. 4. Cow milk, heated for 10 min. at 110°.

Fig. 2. Effect of cysteine on the growth of *L. bifidus* (TM2).

Table 2. *Effect of small additions of milk on the growth of* L. bifidus (*TM* 2 *and KA* 2) *in the basal medium containing cysteine*

Additions to basal medium	Strain TM2	Strain KA2
	ml. 0·1N-NaOH/10 ml. medium	
Nil	1·1	0·8
Cysteine, 80 μg./ml.	3·3	2·9
Ditto + 0·5 ml./10 ml. unautoclaved human milk	10·7	8·3
Ditto + 0·5 ml./10 ml. autoclaved human milk	10·6	7·0
Ditto + 0·5 ml./10 ml. autoclaved cow milk	10·6	7·2

Table 3. *Effect of different additions on the growth of* L. bifidus (*TM* 2) *in the basal medium*

Supplement/10 ml. basal medium	ml. 0·1N-NaOH – blank		
	Exp. 1	Exp. 2	Exp. 3
1. Cow milk (0·2 ml.)	12·8	15·3	12·6
2. Whey (=0·2 ml. milk)	..	14·6	10·2
3. Tryptic digest* of casein separated from milk (=0·2 ml. milk)	6·7	6·9	5·3
4. Tryptic digest of lactalbumin separated from milk (=0·2 ml. milk)	3·4	..	..
5. Tryptic digest of whey (=0·2 ml. milk)	11·1	13·2	10·7
6. Tryptic digest of commercial casein (0·2 ml.)	..	..	7·4
7. Tryptic digest of egg albumin (0·2 ml.)	..	..	14·7
8. Tryptic digest of blood albumin (0·2 ml.)	..	..	14·1
9. Tryptic digest of pepsin (0·2 ml.)	..	..	9·3
10. Tryptic digest of gelatin (0·2 ml.)	..	..	3·5
11. Tryptic digest of zein (0·2 ml.)	..	..	2·0
12. Trypsin solution* (0·2 ml.)	..	..	1·3
13. Mixture of B-vitamins† (0·2 ml.)	0·0	..	0·0
14. Mixture of nucleotides† (0·2 ml.)	0·1	..	0·0
15. (13)+(14) (0·2 ml. of each)	..	..	0·0
16. Tween 80 (0·1 ml.)	..	0·6	..
17. Ash of cow milk (=0·2 ml. milk)	..	0·0	..
18. Mixture of amino-acids† (0·2 ml.)	1·7	..	..

* The tryptic digests were prepared by digesting 3 g. protein with 25 mg. trypsin (1 : 250, Difco) at 37° for 24 hr. The solution was then filtered, and the filtrate diluted with water to 100 ml.; pH value of digests adjusted to 6·8. To test the effect of trypsin 25 mg. was dissolved in 100 ml. water.

† The mixture of B-vitamins had the following composition: 10 mg. thiamine-HCl, 10 mg. riboflavin, 10 mg. nicotinic acid, 25 mg. pyridoxin-HCl, 2 mg. *p*-aminobenzoic acid, 1 mg. folic acid, 100 mg. inositol, 100 mg. choline, and 30 μg. vitamin B_{12} in 200 ml. water. The 'nucleotide mixture' contained adenylic acid, guanylic acid, uridylic acid, xanthine and thymine, 10 mg. of each in 10 ml. water. The amino-acid mixture was composed in such a way as to simulate the amino-acid composition of milk.

hydrolysates of commercial protein preparations were investigated. Tryptic hydrolysates of several proteins were strongly active in promoting the growth of the test strain, while acid hydrolysates of the same proteins were completely inactive. These properties and the heat-stability of the substance (Figs. 3 and 4) show a striking similarity of the active principle to strepogenin (Wright & Skeggs, 1944; Sprince & Woolley, 1945). To obtain further information on this relationship experiments were performed to determine the activity of our preparations by means of the assay for strepogenin which uses *L. casei* (Kodicek & Mistry, 1952).

Effect of different preparations on the growth of L. casei *and* L. bifidus (*TM* 2)

The results of an experiment to show the effect of various protein preparations on the two organisms are given in Table 4. Although the growth-promoting activity of almost all preparations tested was higher with *L. bifidus* than with *L. casei* the results with the two organisms compare fairly closely.

Egg albumin, blood albumin and milk were most active in both cases; gelatin and zein had very low activity. The activity of whey, and tryptic digest of whey or pepsin was much more marked with *L. bifidus.*

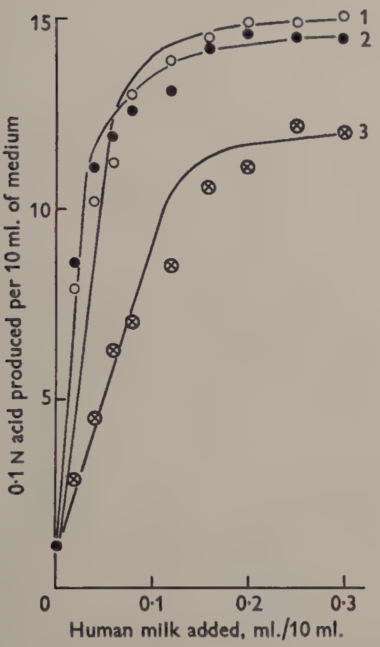


Fig. 3

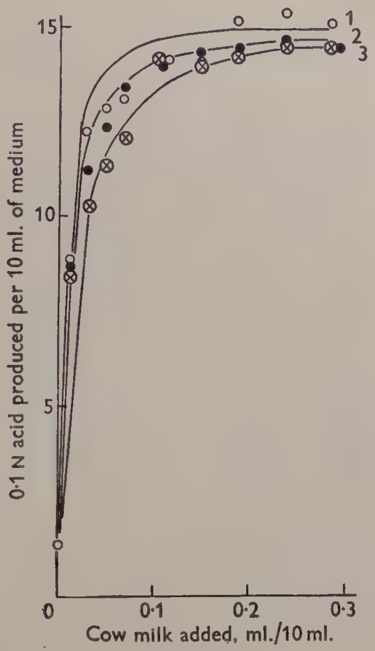


Fig. 4

Fig. 3. Effect of human milk on the growth of *L. bifidus* (TM2) in the basal medium containing cysteine (100 μg./ml.). 1. Human milk, heated for 30 min. at 63°. 2. Human milk, heated for 10 min. at 110°. 3. Human milk whey, heated for 10 min. at 110°.

Fig. 4. Effect of cow milk on the growth of *L. bifidus* (TM2) in the basal medium containing cysteine (100 μg./ml.). 1. Milk, heated for 30 min. at 63°. 2. Milk, heated for 10 min. at 110°. 3. Whey, heated for 10 min. at 110°.

Table 4. *Effect of different preparations on the growth of* L. casei (*ATCC* 7469) *and* L. bifidus (*TM* 2); *order of activities*

L. casei		*L. bifidus*	
Preparation	Acid formation (ml. 0·1N-NaOH)	Preparation	Acid formation (ml. 0·1N-NaOH)
Milk	15·7	Egg albumin, tryptic digest	14·7
Egg albumin, tryptic digest	9·9	Blood albumin, tryptic digest	14·1
Blood albumin, tryptic digest	7·0	Milk	12·6
Whey	3·7	Whey, tryptic digest	10·7
Whey, tryptic digest	3·1	Whey	10·2
Pepsin, tryptic digest	1·6	Pepsin, tryptic digest	9·3
Zein, tryptic digest	1·0	Casein, tryptic digest	7·4
Casein, tryptic digest	0·3	Gelatin, tryptic digest	3·5
Gelatin, tryptic digest	0·0	Zein, tryptic digest	2·0

The strepogenin activity of different preparations has usually been compared with that of an arbitrary liver standard (e.g. Woolley, 1948). Since insulin digests, according to Sprince & Woolley (1945), may be the most potent natural sources of strepogenin, Kodicek & Mistry (1952) proposed insulin as standard in strepogenin assays. Since suitable insulin preparations were not available to the present authors, tryptic digests of egg albumin were used as an arbitrary standard. The activities of a number of preparations were compared with each other. The different protein digests were prepared by hydrolysis with trypsin for 24 hr.; with conc. HCl for 3 hr. at 37°; with conc. HCl for 20 hr. at 37°; and with conc. HCl for 3 hr. at 100°. The digests contained 4 mg./ml. in terms of starting material. The untreated preparations were dissolved at the same concentration and milk and whey were diluted to contain 4 mg. solids/ml. Each preparation was tested at three concentrations. Relative strepogenin activities (tryptic digest of egg albumin as 100) tested with *L. bifidus* TM 2 were found to be: blood albumin, tryptic digest 27; peptonized milk (Bacto-Difco) 22; yeast extract (Bacto-Difco) 22; pepsin, tryptic digest 20; proteose-peptone (Bacto-Difco) 19; milk 17; whey 17; casein, tryptic digest 16; gelatin, tryptic digest 14; trypsin, untreated 14; blood albumin, untreated 13; glutatione 13; peptone (Bacto-Difco) 10; casein, HCl-digest, 3 hr. at 37° 9; pepsin, untreated 9; egg albumin, untreated 8; tryptone (Bacto-Difco) 8; neopeptone (Bacto-Difco) 6; blood albumin, HCl-digest, 3 hr. at 37° 5; casein, untreated 4; casein, HCl-digest, 20 hr. at 37° 3; gelatin, untreated 2; asparagine 2; other acid hydrolysates were completely inactive. The corresponding activities tested with *L. casei* were: Yeast extract 220; milk 74; whey 72; blood albumin, untreated 61; blood albumin, tryptic digest 44; egg albumin, untreated 33. Tryptic and acid digests of casein were inactive in concentrations tested. It can be seen that the activity of milk and whey as compared with that of the tryptic digest of egg albumin was much greater for *L. casei* than for *L. bifidus*. Furthermore, yeast extract and untreated egg- and blood-albumin were more potent sources of the active substance for *L. casei* than for *L. bifidus*. The inactivity of casein preparations for *L. casei* was confirmed in this experiment.

DISCUSSION

The strains of *L. bifidus* studied by the present authors were isolated and subcultivated under conditions reported to maintain *L. bifidus* unchanged. In fact no spontaneous clear-cut changes were observed except that the ability to develop intensive growth in the minimal medium gradually decreased. Nutritional properties characteristic of the straight rod type were not, however, observed during 5 months' work with them. The bifid morphology became less pronounced during subcultivation *in vitro* for some months, although bifid forms were then still present in abundance. It seems that the minimal medium employed (Hassinen *et al.* 1951) permits minor changes in morphology and nutritional properties of the bifid type, but that the change is not to the straight rod type. As shown by Norris *et al.* (1950) and by Hassinen *et al.* (1951) many properties of *L. bifidus* are readily changed by subcultivation *in* *vitro* under certain conditions, the simple nutritional requirements of the bifid type changing to the more complicated requirements of the straight rod type. It seems probable, however, that intermediates between these two main types can also develop from strains of the bifid type, and that the strains of *L. bifidus* described in this paper must be considered as such intermediates. They were similar to the bifid type in most respects but differed from it in their requirement of the essential factor in milk. The growth-promoting factor for *L. bifidus* found in milk, whey, and enzymic protein hydrolysates appeared to be closely related to strepogenin. Except with casein preparations, results from comparative assays with *L. casei* and *L. bifidus* were in good agreement. Our finding of the inactivity of casein hydrolysates for *L. casei* agrees with that of Kodicek & Mistry (1952) who made a similar observation. Wright, Fruton, Valentik & Skeggs (1950), on the other hand, who used *Lactobacillus bulgaricus* as the test organism, recorded fairly high activity values for casein preparations. Again, egg albumin, which in our experiments had the highest activity, according to Wright *et al.* (1950) was only slightly active for *L. bulgaricus*. It appears that the term 'strepogenin' may cover different combinations of amino-acids and thus the requirements for this factor can be different with different strains and species of lactobacilli. The 'strepogenin' activity of milk and whey cannot be ascribed to any particular protein since digests of casein as well as lactalbumin were active. The relatively high potency of whey could be explained by assuming that the active substance is formed during the operations connected with the removal of protein, and is then enriched in the whey.

REFERENCES

Bergey's Manual of Determinative Bacteriology (1948), 6th ed. Ed. R. S. Breed, E. G. D. Murray and A. P. Hitchens, p. 1529. London: Baillière, Tindall and Cox.

Clegg, K. M., Kodicek, E. & Mistry, S. P. (1952). A modified medium for *Lactobacillus casei* for the assay of B-vitamins. *Biochem. J.* **50**, 326.

Hassinen, J. B., Durbin, G. T., Tomarelli, R. M. & Bernhart, F. W. (1951). The minimal nutritional requirements of *Lactobacillus bifidus. J. Bact.* **62**, 771.

Kodicek, E. & Mistry, S. P. (1952). The microbiological assay of 'strepogenin' with *Lactobacillus casei. Biochem. J.* **51**, 108.

Norris, R. F., Flanders, T., Tomarelli, R. M. & György, P. (1950). The isolation and cultivation of *Lactobacillus bifidus.* A comparison of branched and unbranched strains. *J. Bact.* **60**, 681.

Sprince, H. & Woolley, D. W. (1945). The occurrence of the growth factor strepogenin in purified proteins. *J. Amer. chem. Soc.* **67**, 1734.

Teply, L. J. & Elvehjem, C. A. (1945). The titrimetric determination of '*Lactobacillus casei* factor' and 'folic acid'. *J. biol. Chem.* **157**, 303.

Tissier, H. (1899). La réaction chromophile d'Escherich et le *Bacterium coli. C.R. Soc. Biol., Paris*, **51**, 943.

Tissier, H. (1900). *Recherches sur la flore intestinale normale et pathologique du nourrisson.* Thèse: Université de Paris. (Quoted by E. Olsen (1949).) *Studies on the intestinal flora of infants*, p. 147. Copenhagen: Ejnar Munksgaard.

Tomarelli, R. M., Norris, R. F. & György, P. (1949*a*). Inability of vitamin B_{12} to replace the desoxyriboside requirement of *Lactobacillus bifidus. J. biol. Chem.* **179**, 485.

Tomarelli, R. M., Norris, R. F., György, P., Hassinen, J. B. & Bernhart, F. W. (1949*b*). The nutrition of variants of *Lactobacillus bifidus*. *J. biol. Chem.* **181**, 879.

Tomarelli, R. M., Norris, R. F., Rose, C. S. & György, P. (1950). The effect of fatty acids on the growth of strains of *Lactobacillus bifidus*. *J. biol. Chem.* **187**, 197.

Weiss, J. E. & Rettger, L. F. (1938). Taxonomic relationship of *Lactobacillus bifidus* (*Bac. bifidus* (Tissier)) and *Bacteroides bifidus* (Eggerth). *J. infect. Dis.* **62**, 115.

Woolley, D. W. (1948). Strepogenin activity of derivatives of glutamic acid. *J. biol. Chem.* **172**, 71.

Wright, L. D., Fruton, J. S., Valentik, K. A. & Skeggs, H. R. (1950). An improved assay for 'strepogenin' based on essential nature of material for *Lactobacillus bulgaricus* O9. *Proc. Soc. exp. Biol., N.Y.* **74**, 687.

Wright, L. D. & Skeggs, H. R. (1944). The growth factor requirements of certain streptococci. *J. Bact.* **48**, 117.

(*Received* 23 *February* 1953)

Cooper, P. D. (1953). *J. gen. Microbiol.* 9, 199–206.

The Study of Cell Rupture in *Staphylococcus aureus*

By P. D. COOPER

The Wright-Fleming Institute of Microbiology, St Mary's
Hospital Medical School, Paddington, W. 2

SUMMARY: In order to prepare staphylococcal cell walls as free as possible from intact organisms yet with minimum chemical and mechanical degradation, a rapid turbidimetric method was developed to measure the proportion of staphylococci ruptured by shaking with glass beads. The assay took less than a minute, and was used to find the bacterial concentration and speed and time of shaking which gave the biggest yields of cell walls in the shortest time.

When bacterial suspensions are shaken with small glass beads the turbidity decreases (Curran & Evans, 1942) and the viable count steadily drops according to a logarithmic law (King & Alexander, 1948). This appears to be due to mechanical rupture of the organisms, as the cell walls of the burst bacteria can be separated from the soluble cytoplasmic contents by centrifugation (Cooper, Rowley & Dawson, 1949; Salton & Horne, 1951). It has recently been found (Few, Cooper & Rowley, 1952) that when penicillin-sensitive staphylococci are rapidly disrupted in a medium containing formalin ('Medium A', see below) the penicillin-binding component present in the organisms (Rowley, Cooper, Roberts & Lester Smith, 1950) is retained in the cell wall, while rupture in distilled water alone gives inactive cell walls. In order to ascertain the optimum conditions for disruption, a rapid turbidimetric method was developed to estimate the number of bacteria disrupted. It had the advantage of enabling an on-the-spot estimation to be carried out in less than a minute. Thus at least 98% of the organisms could be broken without allowing the suspension to shake or stand more than necessary, so that mechanical and chemical degradation of the cell walls was minimized. The turbidimetric method and some factors affecting rate of rupture of staphylococci are described below.

METHODS

Preparation of bacterial suspensions. The organism used was a Cowan's type II penicillin-sensitive *Staphylococcus aureus* kindly supplied by Dr Hobbs of the Central Public Health Laboratory, Colindale. After growing on beef tryptic digest agar for 16 hr. at 37° the organisms were harvested in distilled water, passed through a No. 1 porosity sintered glass filter funnel to remove agar particles, and washed 3 times with distilled water.

Apparatus used for bacterial disruption. A 'Microid' flask shaker (Messrs Griffin and Tatlock, London) oscillated a maximum load of four shaking tubes (each weighing 100 g. with contents) at a continuously variable frequency of up to 45 cycles/sec., or two tubes up to 53 cycles/sec. The shaking tubes, 140 mm. × 30 mm. and with a row of indentations down each side to ensure turbulence while shaking, were closed by glass stoppers. The tubes contained 20 ml. bacterial suspension and 20 g. glass 'Ballotini' beads (Messrs Chance Bros., Smethwick, England, 0·1–0·2 mm. diam., grade '12'). The tubes were clamped on the shaker in a horizontal position and shaken vertically with an amplitude of 2·5 cm.

The shaking frequency was determined as follows. A bar magnet was clamped to the shaking arm close to the iron core of a solenoid of approximately 2000 coils which was connected to the Y input of an oscilloscope. A calibrated oscillator connected to the X input was set to a known frequency and the speed of the shaker was continuously tuned to this frequency during shaking by keeping the single Lissajou figure stationary. Without constant adjustment the frequency of the shaker was liable to vary by $\pm 0{\cdot}5$ cycles/sec. and a mains drop of 12 volts caused a frequency drop of 1 cycle/sec. The shaker ran at a different speed if not 'warmed up'. At what appeared to be the same adjustment the frequency differed from day to day. These variations were sufficient to prevent the reproducible rates of breakage necessary to find the optimum conditions, but were completely eliminated by using the tuning system described.

Assay of results. Bacterial dry wt. was determined with a Hilger absorptiometer calibrated in terms of mg. dry wt./ml.

The 'turbidity' of suspensions before and after shaking was measured on 0·25 ml. samples diluted to 10 ml. in distilled water contained in 1 cm. cells in the same instrument with neutral filters. For the present purpose 'turbidity' is taken as the 'Spekker' dial reading, $= 2 - \log_{10}$ percentage transmission. The percentage of bacteria ruptured was calculated from the relation between the turbidities of a suspension before and after rupture. The relationship is derived below from the rate of drop in turbidity during rupture.

The number of viable organisms present in suspensions before and after shaking was determined by the method of Miles & Misra (1938). Serial dilutions of the suspension were added to 5 ml. broth in capped bottles which were vigorously shaken before removal of the sample for the next dilution. Five drops of each broth dilution were allowed to fall on separate areas of an agar plate which was then tilted and rotated to spread the drops, and the colonies in each of the five groups formed were counted after 48 hr. incubation at 37°. Dilutions were arranged so that the number of colonies per group was 20–50.

The ratio of whole bacteria to cell walls was also observed directly from electron micrographs of washed suspensions.

Composition of shaking media. 'Medium A' contained in 100 ml.: 1 ml. formalin solution, 0·5 g. citric acid, 50 ml. distilled water saturated with octyl alcohol, 0·1 ml. mercaptoacetic acid (thioglycollic acid). These substances were added respectively to reduce enzyme activity, as buffer, to reduce enzyme activity (Meyerhof, 1949) and frothing, and to reduce possible autoxidation. 'Medium B' contained in 100 ml.: 1 ml. formalin solution, 0·5 g. citric acid. Both media were adjusted to pH 6·5 with 10 N-NaOH solution before final adjustment to volume with distilled water.

RESULTS

Changes of turbidity occurring during shaking

The turbidity in distilled water was found to fall non-linearly with time of shaking and reached a nearly level value which was extrapolated to zero time to a value of about 17·5 % of the initial turbidity in order to correct for the further slow drop in turbidity on continued shaking (Fig. 1). This value was independent of the frequency of shaking and concentration of bacteria, although the rate at which it was approached depended on these factors.

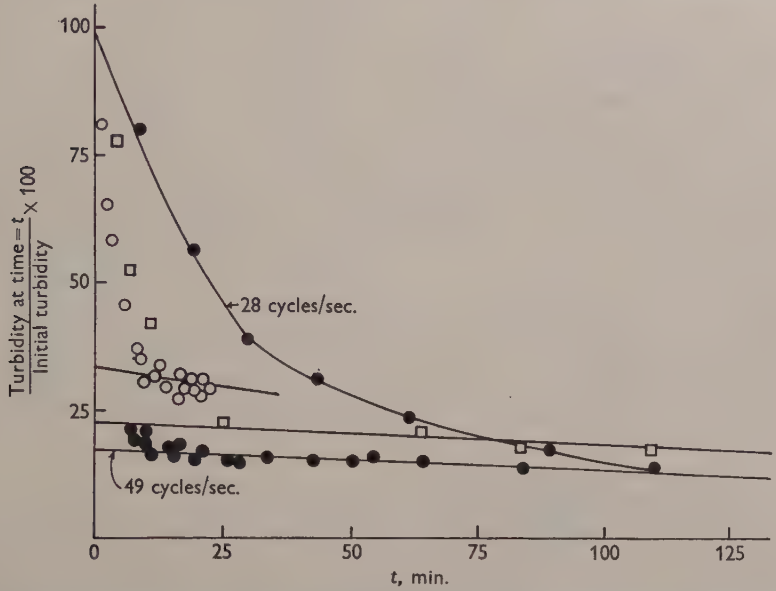


Fig. 1. Limiting turbidity reached on disrupting *Staph. aureus* at 2 mg. dry wt./ml. in distilled water, ●—●, 28 and 49 cycles/sec.; 'Medium B', □—□, 49 cycles/sec.; 'Medium A', ○—○, 53·5 cycles/sec.

In 'Medium A' and 'Medium B' the turbidity fell similarly to level values which were extrapolated to about 33 and 23·5 % respectively of initial turbidity. At these levels electron micrographs, staining by Gram's method, and viable counts where applicable showed that substantially all the bacteria were disrupted. In all experiments the rate of turbidity decrease was less in the presence of formalin than in distilled water alone.

A plot against time of

$$\log_{10}\left[\frac{\text{turbidity at time}=t}{\text{turbidity at time}=\text{zero}}-0{\cdot}175\right]$$

gave a straight line for distilled water, and substitution of 0·175 by 0·235 for 'Medium B' and 0·33 for 'Medium A' also gave straight lines (Fig. 2). The values 0·175, 0·235 and 0·33 were chosen as those giving the best average extrapolations and straight lines for a large number of experiments, but from the range of deviations given in Fig. 2 for two lines it can be seen that they are liable to an error probably of the order of ±0·01 unit. The fact that the values giving the best straight lines corresponded closely with the best extrapolations supports the reasons for the choice of the formula

$$\log\left[\frac{T_t}{T_0}-\text{constant}\right]\propto\text{time}$$

which are discussed below.

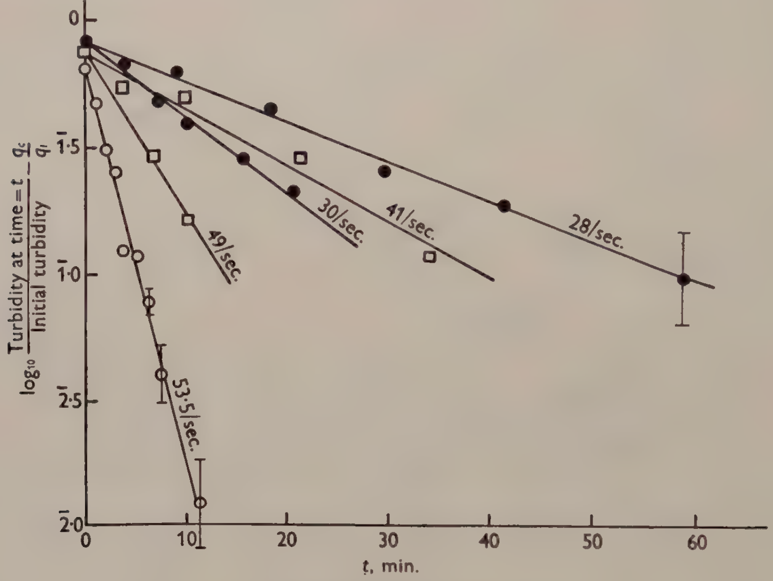


Fig. 2. Relation between $\log_{10}\left[\frac{\text{turbidity at time}=t}{\text{initial turbidity}}-\frac{q_c}{q_i}\right]$ and time of shaking where $\frac{q_c}{q_i}=0\cdot175$, distilled water, ●—●; 0·235, 'Medium B', □—□; 0·33, 'Medium A', ○—○. The deviations shown on two curves represent variations caused by errors of ±0·01 unit in the values for $\frac{q_c}{q_i}$.

Correlation between the percentage of organisms ruptured calculated from turbidity measurements and that observed from viable and electron micrographic counts

It is demonstrated below that in distilled water the percentage of bacteria ruptured, P, after t minutes shaking should be given by the expression

$$\frac{P}{100}\left[1-\frac{q_c}{q_i}\right]=1-\frac{Q}{q_i},$$

where q_i is the initial turbidity, $\frac{q_c}{q_i}=0\cdot175$ and Q is the observed turbidity at time t. Table 1 shows that the number of ruptured organisms calculated by this means from turbidity measurements agrees well with the number deduced from viable and electron micrographic counts.

Table 1. *Correlation between the rates of disruption calculated by means of turbidity measurements and observed from viable and electron micrograph counts. Experiments* 1 *and* 2 *performed on separate days but otherwise identical.* 2 *mg./ml. dry wt. of cells shaken in distilled water at* 40 *cycles/sec.*

Experiment 1			Experiment 2		
	% intact cells from			% intact cells from	
Time (min.)	Viable counts	Turbidity changes	Time (min.)	Electron micrograph counts	Turbidity changes
0	100	100	0	100	100
2	43·3	43·2	1	82	81·6
4	16·2	16·2	3	63·1	57·4
6	9·6	10·2	5	45·7	39·2
8	4·7	4·1	7	31·4	26·4
10	2·6	1·5	9	24·3	19·5
			11	17·4	14·0

Factors affecting rate of rupture

It can be seen from Fig. 2 that in presence of 'Medium B' the rate of rupture is considerably slowed. The rate of rupture is also dependent on frequency of oscillation, so that frequencies less than 30/sec. give a very slow break-up with the apparatus employed (Fig. 3). This rather critical value may be due to the

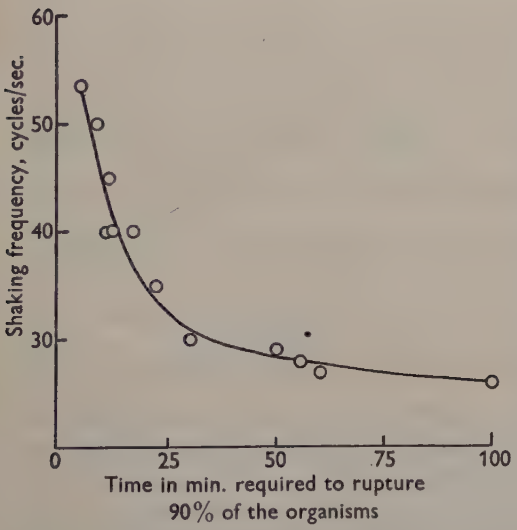


Fig. 3

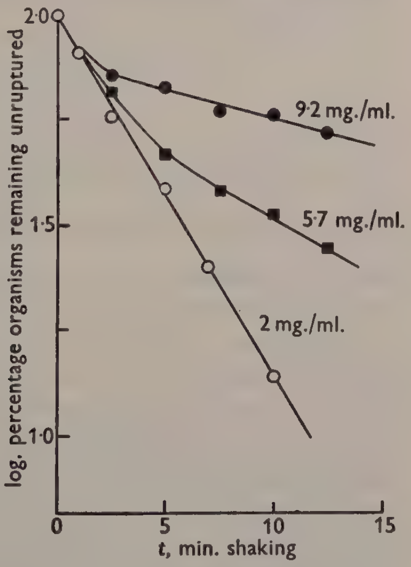


Fig. 4

Fig. 3. Relation between time required to rupture 90% of *Staph. aureus* calculated from turbidity measurements, and frequency of shaking in distilled water at 2 mg. dry wt. of cells/ml.

Fig. 4. Effect of varying *Staph. aureus* concentration on rate of disruption in distilled water calculated from turbidity measurements. Shaken at 40 cycles/sec. 2 mg. dry wt. cells/ml., ○—○; 5·7 mg./ml., ■—■; 9·2 mg./ml., ●—●.

use of vertical shaking, as little movement of the beads will occur unless the acceleration on the downward throw is greater than that due to gravity.

At bacterial concentrations greater than 2–3 mg./ml. the formation of cell walls ceases to follow a logarithmic law, and the rate of rupture falls off (Fig. 4), but below 2–3 mg./ml. the rate is independent of bacterial concentration. This slowing of rupture is probably due to frothing at the higher concentrations.

DISCUSSION

It can be shown experimentally that the sum of the 'turbidities' of suspensions of staphylococci at say Y/ml. and staphylococcal cell walls at Z/ml. is identical with the turbidity of a mixed suspension containing in 1 ml. Y and Z of the respective particles, and also that turbidity is directly proportional to concentration for both suspensions. Therefore the turbidity of a staphylococcal suspension after partial mechanical rupture with glass beads would be expected to consist of the sum of the turbidity due to unchanged cells and the turbidity of the cell walls produced from those bacteria which have been ruptured.

The rate of killing of bacteria by mechanical rupture follows a logarithmic law (King & Alexander, 1948) so that, if complete rupture is the sole cause of killing, the turbidity of a given suspension after t minutes shaking should equal

$$q_i . e^{-t/\lambda} + q_c[1-e^{-t/\lambda}]$$

where q_i = turbidity of the initial suspension, q_c = turbidity of the cell walls produced by 100% rupture of that suspension, and λ is a constant.

Thus Q, the observed turbidity at time t,

$$= q_i . e^{-t/\lambda} + q_c[1-e^{-t/\lambda}] = q_c + [q_i - q_c] . e^{-t/\lambda}.$$

Therefore $$\log\left[\frac{Q-q_c}{q_i}\right] = \log\left[\frac{q_i-q_c}{q_i}\right] - \frac{t}{\lambda}.$$

In this equation Q and t could be determined experimentally, q_i was determined before the start of the experiment and $\frac{q_c}{q_i}$ should be given by the extrapolation of the level values of the turbidities finally reached, as in this case only cell walls were present.

Using these experimental values, a plot of $\log_{10}\left[\frac{Q}{q_i} - \frac{q_c}{q_i}\right]$ against time where $\frac{q_c}{q_i}$ was 0·175, 0·235 and 0·33 for distilled water, 'Medium B' and 'Medium A' respectively, was found to give a straight line. Thus it appeared that the changes in turbidity observed may closely follow the rate of rupture and killing of the organisms. This was confirmed by the close correlation found between the rate of fall of viable counts, rate of rupture observed from electron micrograph counts and the percentage of bacteria broken, P, which can be calculated from the relation

$$Q = q_i . \frac{(100-P)}{100} + q_c . \frac{P}{100}, \quad \text{or} \quad \frac{P}{100}\left[1 - \frac{q_c}{q_i}\right] = 1 - \frac{Q}{q_i}.$$

In practice P is obtained from the straight line graph relating P to $\frac{Q}{q_i}$.

As the rate of fall of viable counts has been found by other workers (King & Alexander, 1948) to be logarithmic, it was assumed above that death only occurs simultaneously with complete rupture in order to deduce a logarithmic fall in turbidity. The fact that the observed results followed the theoretical equations and that viable and electron micrographic counts agreed closely with the production of cell walls calculated by this method confirm this assumption. Thus the staphylococci are able to withstand considerable agitation and only die when completely ruptured. It is not possible to say from this whether partially ruptured organisms are viable, or whether, as appears more likely, the mere splitting of the cell envelope kills the cell and the subsequent complete emptying of the cell contents to leave 'clean' cell walls occurs rapidly.

This correlation between rupture and killing differs from the recent findings of Furness (1952) with *Bacterium coli*, where the total count was always much higher than the viable count. This may possibly be due to the inclusion of partially ruptured organisms in the total count, as in my experience coliforms do not yield 'clean' cell walls on mechanical rupture in as high a proportion as do staphylococci.

The conditions for most rapid rupture obtained from the above data have been described elsewhere (Few *et al.* 1952).

The slow drop in the turbidity of cell wall suspensions on further shaking, which was more marked at higher speeds, seemed likely to be due to the mechanical disintegration of the cell walls which was noticed in electron micrographs of such suspensions. For this reason it was important not to shake longer or faster than necessary when preparing cell walls which were capable of binding penicillin, as the penicillin-binding component was easily removed by the mechanical grinding provided by the Ballotini beads. Formalin appears to protect the cells to some extent against rupture, and the cell walls prepared in 'Medium A' contain more material and appear considerably thicker in electron micrographs than those from distilled water (Cooper, 1953).

A slight turbidity was observed when Ballotini beads were shaken alone in distilled water, due apparently to small splinters of glass. It did not interfere with the results described above, however, as it never amounted to more than ½% of the turbidities measured, and also much less splintering occurred in the presence of the organisms than in their absence. The very small turbidity contributed by the ultramicroscopic 'lipid particles' of Mitchell & Moyle (1951) also liberated on rupture of the organisms would be included in the term q_c.

I wish to express my thanks to London University for a grant for apparatus from the Central Research Fund, and to Dr C. Challice of the National Institute for Medical Research, Mill Hill, London, for electron micrographs.

REFERENCES

COOPER, P. D. (1953). The site of action of penicillin. 2. The association of the penicillin-binding component (PBC) with a lipid fraction in *Staphylococcus aureus*. *Biochem. J.* (in the Press).

COOPER, P. D., ROWLEY, D. & DAWSON, I. M. (1949). Location of radioactive penicillin in *Staphylococcus aureus* after contact with the drug. *Nature, Lond.* **164**, 842.

CURRAN, H. R. & EVANS, F. R. (1942). The killing of bacterial spores in fluids by agitation with small inert particles. *J. Bact.* **43**, 125.

FEW, A. V., COOPER, P. D. & ROWLEY, D. (1952). Reaction of penicillin with the staphylococcal cell wall. *Nature, Lond.* **169**, 283.

FURNESS, G. (1952). Some factors affecting the destruction of *Bacterium coli* by shaking with glass beads. *J. gen. Microbiol.* **7**, 335.

KING, K. H. & ALEXANDER, H. (1948). The mechanical destruction of bacteria. *J. gen. Microbiol.* **2**, 315.

MEYERHOF, O. (1949). The Harden-Young effect in alcoholic fermentation of yeast preparations. *J. biol. Chem.* **180**, 575.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

MITCHELL, P. & MOYLE, J. (1951). Glycerophospho-protein complex envelope of *Micrococcus pyogenes*. *J. gen. Microbiol.* **5**, 981.

ROWLEY, D., COOPER, P. D., ROBERTS, P. W. & LESTER SMITH, E. (1950). The site of action of penicillin. I. Uptake of penicillin on bacteria. *Biochem. J.* **46**, 157.

SALTON, M. R. J. & HORNE, R. W. (1951). Studies on the bacterial cell wall. II. Methods of preparation and some properties of cell walls. *Biochim. Biophys. Acta*, **7**, 177.

(*Received* 2 *March* 1953)

Heald, P. J., Krogh, N., Mann, S. O., Appleby, J. C., Masson, F. M. & Oxford, A. E. (1953). *J. gen. Microbiol.* 9, 207–215.

A Method for Direct Viable Counts of the Facultatively Anaerobic Microflora in the Rumen of a Sheep maintained on a Hay Diet

By P. J. HEALD,* N. KROGH,† S. O. MANN, J. CHRISTINE APPLEBY, FRANCES M. MASSON and A. E. OXFORD

Rowett Research Institute, Bucksburn, Aberdeenshire

SUMMARY: By direct viable counts in deep cultures in a basal nutrient agar, consisting of buffered and very highly clarified rumen liquor, supplemented only with vitamin-free acid hydrolysate of casein, tryptophan, a fermentable carbohydrate and agar, the crude wet rumen contents of a hay-fed sheep has been shown to contain a population of *c.* 10^8 viable and facultatively anaerobic bacteria per g., mostly Gram-positive cocci capable of fermenting a wide range of soluble carbohydrates including starch and inulin but not mannitol. The mannitol-fermenting population is of the order of 10^6 organisms per g., consisting mostly of Gram-negative rods, including coliform bacteria. Addition of reducing substances such as sodium sulphide or mercaptoacetate (thioglycollate) to the basal agar had little or no effect in increasing the counts. An extract of hay made with cleared and sterile rumen liquor did not yield the typical rumen streptococcal flora when counted by this method in raffinose + rumen liquor nutrient agar.

Although most workers with rumen micro-organisms seem to have assumed either that the important fermentative bacteria and protozoa in this very mixed culture must be obligate anaerobes, or that they will not act except under powerful reducing conditions (see, e.g. Hungate (1950); Huhtanen, Rogers & Gall (1952) and previous papers by Gall and associates on the methods for isolating rumen bacteria; Sijpesteijn & Elsden (1952) on methods of using washed suspensions of mixed rumen bacteria for fermentation studies), nevertheless there are good reasons for believing that the rumen normally contains a considerable population of facultative anaerobes which are active in fermenting carbohydrates. Thus, by a simple enrichment technique not carried on for more than 18 hr., Heald (1952*a*, *b*) isolated numerous pure cultures of coliform bacteria, capable rapidly of fermenting glucose and xylose, from the rumens of hay-fed sheep. MacPherson (1953), in a study of rumen amylolytic bacteria, without any difficulty isolated numerous facultatively anaerobic streptococci, which resembled *Strep. bovis* in fermentation reactions, from the rumens of both concentrate-fed and hay-fed sheep. These bacteria were absent from the fodder. Sugden & Oxford (1952) showed that an important and ubiquitous group of sheep's rumen ciliate protozoa, the holotrichs, which are very active in fermenting glucose (Heald & Oxford, 1953), are not at all exacting in their demands for anaerobic conditions and will flourish in a liquid buffer containing no reducing substances, when no more drastic measures are taken to exclude air than the fitting of a Bunsen valve to the mouth of the culture flask. In the work here described we have endeavoured to count and characterize the viable facultative anaerobic bacteria in the rumen contents of a hay-fed sheep, using for the diluent a medium containing cleared and sterilized rumen liquor supplemented only with a vitamin-free acid hydrolysate of casein, tryptophan, a fermentable sugar and agar. Since the nature of the fermentable sugar greatly influenced the kind and numbers of bacterial colonies which developed, we extended the investigation to include the whole range of soluble carbohydrates normally used in bacteriology and also some others, found in grass or likely to occur in the rumen, which are not usually employed in fermentation tests.

---

* Present address: Biochemical Laboratories, Institute of Psychiatry, Denmark Hill, London, S.E. 5.

† Present address: Norwegian Veterinary College, Oslo, Norway.

METHODS

Source of rumen contents. For the bacterial counts, rumen contents (*c.* 100 ml.) were taken as required from hay-fed sheep no. 879 (previously used by Sugden & Oxford, 1952) which had a closed permanent rumen fistula. Sampling by means of a wide-bore glass tube was usually carried out at 10 a.m., 2 hr. after feeding; the specimen in a closed flask was immediately placed in the incubator. For preparation of cleared rumen liquor for the basal medium, rumen contents (1 l. at a time) were withdrawn from any available fistulated sheep fed on meadow hay only, or the whole rumen contents of a slaughtered hay-fed sheep were worked up without loss of time. It is much more difficult, or impossible, to obtain a clear rumen liquor from sheep fed on concentrates as well as hay. Hence it has not yet been possible to study the rumen microflora of such sheep by these methods.

Preparation of a clear rumen liquor for use in the basal medium. The coarse plant particles were removed from rumen contents by straining through several layers of surgical gauze. The filtrate was put through a Sharples supercentrifuge, then filtered again through Seitz clarifying and sterilizing pads (Messrs J. C. Carlson Ltd., Ashton-under-Lyne, Lancs) in that order. If the liquor became alkaline at any stage, particularly before Seitz filtration, glacial acetic acid was carefully added to neutralize again. The clear golden-brown liquid was finally stored in the cold room at +2° in sterilized screw-capped bottles 'medical flats'. This clarified rumen liquor would usually keep for a month without showing a precipitate or any sign of growth of micro-organisms; as much as 2–3 l. per week could be prepared without difficulty.

Preparation of a nutrient agar containing the carbohydrate to be tested in cleared rumen liquor supplemented with amino-acids. As described later, viable counts were made in screw-capped 'Universal' bottles (8 × 2·7 cm.) each containing about 12 ml. agar. They were chosen because they are cheap, sturdy and the solidified agar could easily be removed from them aseptically when required without breaking the bottle. The agar contained, per 12 ml.: 5% casein hydrolysate solution (Allen and Hanbury, vitamin-free for microbiological assay), 2 ml., 0·5% DL-tryptophan solution, 0·5 ml.; 1·5% sugar solution, 1 ml.; 2% agar (neutral, Baird and Tatlock Ltd.) dissolved in cleared and buffered rumen liquor, 8·5 ml. Everything was sterilized by steaming, or where possible by Seitz filtration; nothing save glassware was autoclaved. The stock solutions of casein hydrolysate and tryptophan were sterilized by steaming, kept in the cold room and briefly re-steamed before use. A fresh lot of each was prepared every fortnight. The sugar solution was usually sterilized by Seitz filtration, otherwise, with more stable carbohydrates like starch and the sugar alcohols, by steaming.

In order to prevent any appreciable change of pH value during sterilization it was found advisable to buffer the cleared rumen liquor by the addition of 1 ml. phosphate solution/100 ml. cleared rumen liquor; the phosphate solution used contained KH_2PO_4, 10 g.; K_2HPO_4, 15 g.; distilled water, 100 ml. It was also found to be absolutely necessary to add the extra amino-acids to the rumen liquor agar. Cleared rumen liquor itself (from hay-fed sheep) contained too little organic-N to promote quick growth of saccharolytic rumen bacteria.

Setting up the count. Three parallel sets of bottles were always incubated side by side, viz. (1) nutrient agar with the carbohydrate to be tested, (2) with mannitol as positive control, (3) without any added carbohydrate. About 250 ml. clear nutrient agar were required for each experiment. Each 1 oz. screw-capped bottle bore three scratch-marks corresponding to 10, 12, and 14 ml. contents respectively. Some twenty bottles were first sterilized empty on each occasion.

The requisite total amount of agar was melted in the buffered rumen liquor by careful heating over a wire gauze with an asbestos centre. The liquid was then strained through glass wool into another container in the steamer, and sterilized by steaming for 1 hr. on the first day, and 20 min. on each of two succeeding days. The final stages of the last sterilization were carried out in a tall sterile beaker, nearly filled with the molten agar, which after steaming was allowed to cool in the steamer. Usually the dross which had accumulated during the three steamings readily sank to the bottom while the agar was still molten. The clear supernatant was then very carefully decanted into a sterile empty flask, a small portion removed for determination of pH value, and the rest (when bromothymol blue showed that the pH value was between 6 and 7) used immediately to fill the bottles to the 12 ml. mark in each instance. The bottles had previously been charged with the requisite amounts of sterile casein hydrolysate, tryptophan and the solution of the sugar to be tested (or mannitol solution or sterile water). After gentle shaking to effect mixing, the bottles were placed in a water-bath at 45° until required.

The flask containing the rumen contents upon which the count was to be made was then vigorously shaken by hand and one or two standard drops (*c.* 50–60 mg.) were added from a sterile Pasteur pipette to 12 ml. of molten agar in one of the bottles containing extra amino-acids but no carbohydrate. After gentle shaking, 2 ml. were transferred by pouring, i.e. from the 12 ml. mark to the 10 ml. mark, to a second bottle. This second bottle, which now contained 14 ml. agar, was used to start the three series of dilutions: (1) with the carbohydrate to be tested (14→12 ml.); (2) with mannitol (12→10 ml.); (3) with no carbohydrate (10 ml.→8 ml. by guesswork; nothing ever grew in 3 days in these 'no carbohydrate' controls). Each series was likewise carried on for four 7-fold dilutions in all, corresponding to dilutions of *c.* 1/10,000, 1/70,000, 1/500,000, and 1/3 million, respectively, of the original rumen contents. Controls (1 bottle each) were also put up with nutrient agar with the carbohydrate, with mannitol, and with no carbohydrate at all, but without any rumen contents. Nothing ever grew in these uninoculated bottles. When the whole series had been put up in this way, all the bottles were quickly cooled in cold water till the agar had completely set. Each bottle was then completely filled with sterile water, the caps screwed down tightly and the whole incubated at 38° for 4 days in all, colonies evident to the naked eye being counted daily. At the end the water was poured out completely (it usually contained sufficient bacterial growth to be turbid) and the solid agar plug bisected or trisected lengthwise with a sterile spatula. Each piece was then carefully fished out into a sterile dish for preparation of smears or pure culture isolations from individual colonies. Some trials with a sterile liquid paraffin layer above the agar, instead of water, gave trouble in the subsequent examination of individual colonies, because of tiny oil droplets. It was concluded that water was preferable to oil. Trials in which resazurin was incorporated in the agar showed that although the resazurin was not in the reduced state at the start of the incubation, as soon as growth occurred, either in the water above, or in the agar below, it was quickly decolorized. The depth of agar in each bottle was *c.* 3 cm. Thus there would seem to be no reason why obligate anaerobes should not grow in the depths after a day or two, provided that facultative anerobes had begun to grow first of all. Trials in which extra S-containing reducing substances were incorporated in the nutrient agar are described below. The actual weight of rumen contents used in each count was determined by weighing 10 drops of rumen contents delivered by the same Pasteur pipette as used for inoculating the first bottle.

The counting of colonies before dissection of the agar was done with the bottle held in front of a strong light. Only when the agar was quite clear could all the colonies be counted. Hence the necessity for going to great trouble to ensure this, as previously described.

Carbohydrate substrates. Unless otherwise stated, carbohydrates incorporated in the nutrient rumen liquor and other agars were best quality commercial specimens supplied by L. Light and Co., or by Messrs T. Kerfoot (Biochemical Reagent Quality). Amylose was a specimen of purified potato amylose supplied by Dr P. N. Hobson; bacterial levan was the specimen used by Masson & Oxford (1951); rumen holotrich 'starch' was the specimen prepared by these authors (1951). Both these last named carbohydrates may be regarded as possible substrates made by microbiological action in the rumen.

A soluble hemicellulose substrate was obtained from unbleached and untreated flour by aqueous extraction by the method of Perlin (1951). The cold water soluble fraction was purified by ethanol precipitation and the pure white powder so obtained dissolved in a little dilute caustic soda. The solution which was starch-free was diluted to the required carbohydrate concentration (1·5 %) cautiously neutralized with dilute HCl, and sterilized by two brief steamings. According to Perlin pentoses are the chief constituent sugars of this complex, but we have not checked this with our product, save to show that it gave a strong pentose test with HCl and phloroglucinol. It contained some protein as well as polysaccharide.

Isolation of pure cultures from individual colonies developing below the surface of the agar in the counts. By means of a small loop portions of single colonies in the agar from the bottles were transferred to glucose peptone yeast extract broth, and incubated overnight (not longer, unless nothing grew). The liquid culture was then plated out, by streaking on the same medium solidified with agar. Usually only one kind of colony grew on each plate. Single colonies were then transferred to Robertson's cooked meat medium, and after incubation for 24 hr., the cultures were kept in the cold room until required. When it was desired to investigate them further, they were streaked on blood agar, and any plates obviously showing a mixed culture were discarded. Single colonies were then again picked off into Robertson's cooked meat medium. These served as stock cultures. For study of fermentation reactions, inoculations into the carbohydrate broths were made from a culture in nutrient heart broth. Other tests were made by direct inoculation from the cooked meat stock cultures.

It was always desirable to test an isolate in the simple rumen liquor basal agar used for the counting, in order to find out whether it would still grow in presence of the carbohydrate which had been used in the dilution series from which it had been isolated. Some of the isolates from the mannitol agars, for example, did not respond to this test. This may mean that the original colony which developed in the mannitol rumen liquor agar contained more than one bacterial species. There can be little doubt however that the actual culture isolated did originate from rumen contents; so no distinction is made at the moment on these grounds, since the main purpose of this paper is to survey, if possible, the whole facultatively anaerobic rumen population.

RESULTS

Relative counts with various carbohydrate substrates

Only colonies clearly visible to the naked eye, or by means of a ×3 hand-lens were counted. Any background of microscopically small pin-point colonies was ignored. The count on mannitol was always small and of the order of 10^6 organisms/g. rumen contents. The colonies on this mannitol-containing medium usually needed 3 days to reach a diameter of *c.* 1 mm. Many simple sugars gave a very much larger count than did mannitol, the colonies developing in 1 or 2 days. Gas-forming colonies sometimes developed in the mannitol agar, but very rarely on any other carbohydrate. The small compact streptococcal colonies were easily distinguished from others much larger and diffuse in outline and which usually yielded Gram-negative rods. Table 1 summarizes the counts obtained with several carbohydrates and also gelatin, as substrates.

It was always found that the colonies seemed to be evenly distributed throughout the agar. With those sugars which gave a count of the order of

10^8 organisms/g. rumen liquor, both the 5th and the 6th dilutions were counted (if possible) and equal weight given to each. Usually the 5th bottle contained between 5 and 15 times as many colonies as the 6th. Whenever a large count was obtained, the colonies in the 6th bottle were all, or almost all, Gram-positive cocci.

Table 1. *Viable counts of facultatively anaerobic bacteria in the rumen liquor capable of fermenting various carbohydrates*

Period of incubation of carbohydrate agar (days)	Carbohydrate tested	No. of colonies developing (millions/g. rumen contents)		Period of incubation of mannitol agar (days)
		In carbohydrate agar	In mannitol agar	
4	amylose	187	<1	3
4	arabinose	7	1·5	4
2	cellobiose	176	<1	2
3	dulcitol	<1	<1	3
3	galactose	16	2	3
2	glucose	97	<1	3
1	inulin	184	<1	2
3	lactose	30	<1	3
4	levan (bacterial)	50	2·5	3
1	maltose	250	<1	3
2	soluble flour hemicellulose	53	—	—
2	raffinose	120	<1	2
2	rhamnose	2	1·5	2
1	salicin	464	<1	3
4	sorbitol	11	<1	2
2	starch (holotrich)	81	<1	2
1	sucrose	76	2	3
3	xylose	14	3	3
7	gelatin	0	<1	3

Pure cultures isolated from the counting bottles

A large number of representative isolates were obtained in pure culture, and included 142 strains of streptococci. Table 2 gives a picture of the relationship between carbohydrate fermented and the kind of bacteria which developed. The streptococcal isolates from sugars other than mannitol usually fermented starch, but not mannitol; only a small proportion of them had the characteristics of *Strep. faecalis*. A complete description of these and other isolates will be given in a later paper. It may be mentioned here that for a 'fodder control' the hay fed to the sheep was finely chopped and incubated with clear and sterile rumen liquor (5 parts) for 1–2 hr. at 37° and the resulting 'artificial' rumen contents, after vigorous shaking, was counted in the usual way, raffinose and mannitol being the substrates. In each case the count was of the order of 10^6 organisms/g., mostly consisting of Gram-negative rods. The Gram-positive cocci, which developed when genuine rumen contents were counted in raffinose agar (see Table 2) were not in evidence. It cannot yet be stated with certainty whether or not the Gram-negative rods obtained from 'genuine' and from 'artificial' rumen contents are similar.

Table 2. *List of kinds of bacteria actually isolated in pure culture in the viable counts carried out with rumen contents at various dilutions in carbohydrate agars*

(Dilutions: $a=1/10^4$; $b=1/70{,}000$; $c=1/500{,}000$; $d=1/3{,}000{,}000$)

	Total no. of strains isolated in pure culture at various dilutions		
Carbohydrate added to agar for the count	Coliform bacteria*	Streptococci	Other bacteria isolated in pure culture from dilutions *a*, *b* or *c*
Mannitol	Mostly $(a+b)=100$	Mostly $(a+b)=63$	Other Gram-positive cocci : 12 Gram-negative cocci : 3 Gram-positive rods : 4
Galactose	$d=0$	$d=5$	0
Sucrose	$d=0$	$d=11$	Tetracoccus : 1
Lactose	$d=0$	$d=6$	0
Maltose	$d=0$	$d=19$	0
Rhamnose	$b=12$	$b=0$	0
Salicin	$d=0$	$d=19$	Other Gram-positive cocci : 2
Xylose	$a=30$	$a=2$	Gram-negative cocci : 9 Other Gram-positive cocci : 2
Inulin	$d=0$	$d=1$	Other Gram-negative rods† : 5
Raffinose	$d=0$	$d=6$	Other Gram-positive cocci : 4 Other Gram-negative rods† : 10
Amylose	$d=0$	$d=6$	0
Levan (bacterial)	$(b+c)=0$	$(b+c)=4$	Other Gram-negative rods† : 1
Sorbitol	$a=12$	$a=0$	0

* Gram-negative rods producing acid and gas in MacConkey's broth.

† Do not produce acid and gas in MacConkey's broth.

Effect of addition of reducing substances to the basal rumen liquor agar

The effects of the following additions were tested: Na_2S (0·02 %); cysteine hydrochloride (exactly as recommended by Huhtanen *et al.* 1952); Na mercaptoacetate (thioglycollate; 0·002 M). In no case did an appreciably larger number of visible colonies develop than in the bottles without the reducing supplement. The mercaptoacetate at the concentration used seemed to be slightly toxic in that the colonies (with glucose as carbohydrate substrate) took longer to develop, although the final count was the same. Incubation of the bottles in an anaerobic jar likewise had no great effect, save in increasing the 'background' of microscopically small colonies developing during 3 days' incubation.

DISCUSSION

In the main these results confirm the studies of MacPherson (1953) who found a facultatively anaerobic streptococcal population in the sheep's rumen with fermentation reactions similar to those of *Streptococcus bovis*. In view of the experience of previous investigators, it is not clear to us why the addition of reducing substances had so little effect on our viable counts, when a basal agar was used which so nearly resembled rumen liquor itself. Our technique would not, however, count all viable saccharolytic bacteria which need a definite concentration of bicarbonate ions in the medium as well as reducing conditions before growth can occur at all. Opinions may differ on what the order of magnitude of the total viable bacterial population of the rumen of a hay-fed sheep really is. In view of the rather small protein content of the uncleared rumen liquor of sheep fed on meadow hay (*c.* 3·5 mg./g.; McDonald, 1952) it would seem best to adopt for the moment the smallest total count so far reported for that fluid ($1–2 \times 10^9$ organisms/ml.) by Louw & Van der Wath (1943) (although their sheep was admittedly fed on a poor quality veldt hay), rather than the very much higher totals, reported for sheep rather differently fed, by Australian and American workers. Granting this, the population of *c.* 10^8/g. which we find for facultatively anaerobic bacteria capable of fermenting certain soluble carbohydrates (e.g. glucose, maltose, cellobiose, salicin among others in Table 1), is clearly not a negligibly small part of the total viable rumen population. This in turn must necessarily be rather less than the total count. Since streptococci and coliform bacteria, as well as clostridia, flourish in the caecum and large intestine of many animals, there can of course be no *a priori* reason why facultatively anaerobic bacteria should not be present and active in large numbers in the rumen. Preoccupation with cellulose fermentation in that site, which undoubtedly is an anaerobic process, may have blinded some investigators to the fact that many other fodder carbohydrates, including soluble ones like sucrose and grass levans are necessarily often fermented in large amounts in the rumen, and that there is no reason at all to suppose that these other carbohydrate fermentations are brought about in the main by obligatory anaerobic bacteria. The rumen cellulolytic anaerobes, according to Hungate (1950), would certainly not ferment them. Even when the fodder consists entirely of hay, which contains very little truly soluble carbohydrate, there is, as our results show, a not inconsiderable facultatively anaerobic and saccharolytic bacterial population in the sheep's rumen. It would be strange indeed if, when the fodder consists chiefly of soluble substances, e.g. spring grass, the facultatively anaerobic population were not of greater density still. If it should prove possible to obtain a clear rumen liquor in such instances, the supplementation by amino-acids, as in the present work, might not be necessary since the concentration of organic-N would be much greater than in rumen contents from hay-fed sheep (cf. McDonald, 1952).

REFERENCES

Heald, P. J. (1952*a*). The fermentation of pentoses and uronic acids by bacteria from the rumen contents of sheep. *Biochem. J.* **50**, 503.

Heald, P. J. (1952*b*). The metabolism of glucuronic acid by xylose-fermenting coliform bacteria. *Biochem. J.* **52**, 378.

Heald, P. J. & Oxford, A. E. (1953). Fermentation of soluble sugars by anaerobic holotrich ciliate protozoa of the genera *Isotricha* and *Dasytricha*. *Biochem. J.* **53**, 506.

Huhtanen, C. N., Rogers, M. R. & Gall, L. S. (1952). Improved techniques for isolating and purifying rumen organisms. *J. Bact.* **64**, 17.

Hungate, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bact. Rev.* **14**, 1.

Louw, J. G. & van der Wath, J. G. (1943). The influence of varying maize supplements on the digestibility of the cellulose in a poor veld hay in relation to bacterial population of the rumen of sheep with a note on the nitrogen metabolism. *Onderstepoort J. vet. Sci.* **18**, 177.

MacPherson, M. J. (1953). The isolation and identification of amylolytic streptococci from the sheep's rumen. *J. Path. Bact.* **66**, 95.

Masson, F. M. & Oxford, A. E. (1951). The action of ciliates of the sheep's rumen upon various water-soluble carbohydrates including polysaccharides. *J. gen. Microbiol.* **5**, 664.

McDonald, I. W. (1952). The role of ammonia in ruminal digestion of protein. *Biochem. J.* **51**, 86.

Perlin, A. S. (1951). Isolation and composition of the soluble pentosans of wheat flour. *Cereal Chem.* **28**, 370.

Sijpesteijn, A. K. & Elsden, S. R. (1952). The metabolism of succinic acid in the rumen of the sheep. *Biochem. J.* **52**, 41.

Sugden, B. & Oxford, A. E. (1952). Some cultural studies with holotrich ciliate protozoa of the sheep's rumen. *J. gen. Microbiol.* **7**, 145.

(*Received 2 March* 1953)

Wildy, P. & Hare, R. (1953). *J. gen. Microbiol.* 9, 216–225.

The Effect of Fatty Acids on the Growth, Metabolism and Morphology of the Anaerobic Cocci

By P. WILDY and R. HARE

Department of Bacteriology, St Thomas's Hospital Medical School, London

SUMMARY: The sodium salts of a number of fatty acids had little, if any, effect on the metabolism or morphology of representative strains of anaerobic cocci of groups I, II, V and VI (Hare, Wildy, Billett & Twort, 1952). However, strains of groups III and IV in the presence of an adequate concentration of fatty acid showed much greater activity than in media without substances of this nature; growth was very much better, gas was formed in larger amounts and organic acids (e.g. pyruvate) or carbohydrates (e.g. fructose) were attacked. The morphology of these group III and IV organisms was also affected in that the cocci were small (0·5–0·8μ.) and well formed in the presence of adequate concentrations of fatty acid, whereas in control preparations they were larger (1·2–1·6μ.) and sometimes had transverse septa.

In the course of investigations on the effect of various substances on the growth of anaerobic cocci, it was found that Tween 80 stimulated the growth of some strains of these organisms. Further experiments showed that a number of fatty acids also stimulated growth, and had marked effects on the morphology of the organisms and on their ability to ferment carbohydrates. These experiments form the subject of this communication.

EXPERIMENTAL METHODS

Strains studied. The collection and maintenance of the anaerobic cocci used was described by Hare *et al.* (1952). All the strains had been classified by their methods and representative strains were used for the tests.

Apparatus. Because of the danger that ordinary washing methods might fail to remove fatty acids from tubes used in previous experiments, all glassware was soaked in chromic + sulphuric acid for 24 hr. after cleaning and then washed with distilled water. There was also a danger that at oven temperatures fatty substances present in cotton-wool might contaminate the glassware (Wright, 1934). For this reason previously dry-sterilized tubes were not used; instead, clean but unsterile tubes were filled with medium, plugged and sterilized by autoclaving or steaming.

Media. The basal medium employed consisted of: 2% (w/v) peptone (Evans); 0·2% (w/v) yeast extract (Difco); 0·1% (w/v) sodium mercaptoacetate (thioglycollate). After solution in distilled water and the addition of fatty acids, sugars or organic acids as required, sufficient N-NaOH was added to bring the medium to the required pH value. The solution was then brought to boil, immediately filtered through hard filter paper and tubed in 4 ml. quantities in $5 \times \frac{1}{2}$ in. tubes. When the medium contained carbohydrate, each tube was capped with hot vaseline and the batch sterilized in the steamer on three successive days. When salts of organic acids only were added, the tubes were autoclaved at 15 lb./sq. in. for 30 min. and immediately capped with hot vaseline.

When the effect of fatty acids was under investigation the above medium was made with the volume of distilled water decreased by one-tenth, and made up to one volume by the addition of fatty acid solution (or distilled water for controls). The fatty acids were made up as 0·01 M-solution in 0·01 N-sodium hydroxide and held at 100° in a boiling water bath until homogeneous. While still hot, tenfold dilutions were made in distilled water and one volume of these dilutions was added to 9 volumes of medium as described above.

Inoculation of tubes, measurements of opacity, gas formation and final pH *value.* The methods employed were those described by Hare *et al.* (1952), the cultures being incubated for 7 days, the opacity determined on the scale of Brown & Kirwan (1914–15), the length of gas column recorded in millimetres and the final pH value determined colorimetrically on porcelain tiles.

RESULTS

An initial experiment with the addition of Tween 80 showed that while the strains of groups I, II, V and VI (Hare *et al.* 1952) were unaffected by concentrations of this substance ranging from 0·001 % (w/v) to 10 % (w/v), those of groups III and IV grew much more profusely in the higher concentrations. A similar experiment was then carried out with representative strains of all the groups in broth containing varying concentrations of sodium oleate and 1 % pyruvate to stimulate growth (Hare *et al.* 1952). The results are given in Table 1. There was no detectable effect on the strains of groups V and VI, and the only action on group I strains was slight inhibition of gas formation in the highest concentration of oleate. There was also some inhibition of growth and a slight diminution in the quantity of gas produced by group II strains. But the most marked effect was on the strains of groups III and IV. In the case of group III, one strain grew very much better and produced considerably greater quantities of gas in the presence of oleate, particularly at 100 μM. The second strain also produced larger quantities of gas, and although the opacity reading on the 8th day was of the same order as that of the control tube, this was to some extent misleading because opacities of 5 or 6 were actually obtained on the 4th day with this strain. It must be presumed that the lower reading on the 8th day was due to autolysis of the organisms with continued incubation. This phenomenon has repeatedly been observed with this strain. The effect of sodium oleate on strains of group IV was more dramatic, particularly at 100 μM, which caused marked stimulation of the growth of all strains and the production of gas by some of these strains, a character not previously observed with strains of this group.

A similar experiment was then carried out, representative strains from each of the groups being inoculated into media containing 1 % pyruvate and different concentrations of the sodium salts of purified capric, lauric, myristic, palmitic, stearic or oleic acids. The results in so far as they concerned groups I, II, V and VI showed that none of the fatty acids at concentrations from 1 μM to

Table 1. *The behaviour of representative strains of 6 groups of anaerobic cocci in different concentrations of sodium oleate in 1% (w/v) pyruvate broth*

Group	No. of strains tested		Oleate concentration				
			0	1 μM	10 μM	100 μM	1000 μM
I	2	Gas:	2, 17–20	2, 17–20	2, 18–20	2, 21–22	2, 0–17
		Opacity:	2, 3	2, 3	2, 3	2, 3	2, 3
II	2	Gas:	2, 55–80	2, 60–72	2, 72–85	2, 50–70	2, 35–39
		Opacity:	2, 4	2, 4	1, 2 1, 4	1, 2 1, 4	1, 3 1, 4
III	2	Gas:	2, 35–38	2, 30–45	2, 52–87	2, 45–77	2, 31–54
		Opacity:	1, <1 1, 1	1, <1 1, 2	1, 1 1, 5	1, <1 1, 6	1, <1 1, 6
IV	8	Gas:	8, 0	8, 0	8, 0	5, 0	6, 0
		Opacity:	8, <1	7, <1 1, 1	6, 1 2, 2	3, <1–18–22 1, 3 4, 4 2, 5 1, 6	2, 11–20 1, 3 4, 4 1, 5 2, 6
V	2	Gas:	2, 68–71	2, 71–72	2, 70–74	2, 70–77	2, 0–78
		Opacity:	2, 6	2, 6	2, 6	2, 6	2, 6
VI	2	Gas:	2, 0	2, 0	2, 0	2, 0	2, 0
		Opacity:	1, <1 1, 1	2, <1	2, <1	1, <1 1, 1	2, <1

In this table and the succeeding one, the figures relating to 'gas' denote the length (in mm.) of the column of gas collecting between the vaseline seal and the surface of the medium. The figure before the comma denotes the number of strains and the numbers after the readings obtained in this particular experiment.

Those relating to opacity denote the tube on Brown & Kirwan's scale whose opacity was the nearest to that obtained. The figure before the comma denotes the number of strains and that after the comma, the opacity reached in this experiment.

1000 μM had any marked effect on the growth of the strains; but there were marked effects on strains of groups III and IV (see Table 2). Both strains of group III were stimulated by all the compounds except caprate and palmitate. Caprate had no detectable action on strains of group IV, whereas laurate, stearate and oleate were extremely active, with palmitate and myristate occupying an intermediate position. In other experiments, the same strains, together with representative strains of the other groups, were tested under similar conditions in the presence of sodium salts of formic, acetic, propionic, butyric, valeric, caproic, heptoic, caprylic, pelargnonic, margaric, nonadecylic or behenic acids (purity and freedom from contamination by other fatty acids not guaranteed). With compounds containing fewer than 9 carbon atoms per molecule, no effects were obtained, but some stimulation of strains of groups III and IV occurred in the presence of pelargonate, nonadecylate or behenate.

Thus it is clear that a suitable concentration of certain fatty acids is needed if good growth of strains of groups III and IV is to be obtained. Although compounds having this effect possess 12 to 18 carbon atoms per molecule, there was no evidence that their activity is linked with the length of their chains; laurate with 12 carbon atoms and stearate with 18 carbon atoms were

Table 2. *Growth and gas formation of strains of groups III and IV in different concentrations of the sodium salts of purified fatty acids*

	Group III (2 strains) Concentration of fatty acid					Group IV (8 strains) Concentration of fatty acid				
	0	1 μM	10 μM	100 μM	1000 μM	0	1 μM	10 μM	100 μM	1000 μM
CONTROL										
Gas:	2, 28–38	—	—	—	—	8, 0	—	—	—	—
Opacity:	1, <1 1, 1	—	—	—	—	8, <1	—	—	—	—
CAPRATE										
Gas:	—	2, 16–35	2, 14–18	2, 10–14	2, 9–20	—	8, 0	8, 0	8, 0	8, 0
Opacity:	—	1, <1 1, 1	2, <1	2, <1	2, <1	—	8, <1	8, <1	8, <1	8, <1
LAURATE										
Gas:	—	2, 25–40	2, 24–48	2, 50–92	2, 10–17	—	8, 0	8, 0	5, 0 3, 6–18–26	5, 0 3, <1–4–26
Opacity:		1, <1 1, 1	1, <1 1, 3	1, <1 1, 6	1, <1 1, 6	—	8, <1	6, 1 2, 2	2, 2 1, 3 2, 4 3, 5	1, 1 1, 2 1, 3 4, 4 1, 6
MYRISTATE										
Gas:	—	2, 29–35	2, 20–34	2, 44–64	2, 5–59	—	8, 0	8, 0	7, 0 1, <1	7, 0 1, 6
Opacity:	—	1, <1 1, 1	1, <1 1, 2	1, 3 1, 4	2, 2	—	8, <1	6, <1 2, 1	5, <1 2, 2 1, 3	5, <1 1, 1 1, 2 1, 5
PALMITATE										
Gas:	—	2, 18–35	2, 12–34	2, 5–11	2, 1–1	—	8, 0	8, 0	8, 0	8, 0
Opacity:	—	1, <1 1, 1	1, <1 1, 1	1, <1 1, 1	1, 1 1, 2	—	8, <1	8, <1	3, <1 5, 1	1, <1 1, 1 5, 2 1, 3
STEARATE										
Gas:	—	2, 29–35	2, 12–30	2, 27–74	2, 15–22	—	8, 0	8, 0	8, 0	8, 0
Opacity:	—	1, <1 1, 2	1, <1 1, 2	1, 2 1, 3	1, 3 1, 4	—	8, <1	4, <1 4, 1	3, 1 3, 2 2, 3	5, 4 3, 5
OLEATE										
Gas:	—	2, 30–45	2, 52–87	2, 55–77	2, 31–54	—	8, 0	8, 0	5, 0 3, <1–18–22	6, 0 2, 11–20
Opacity:	—	1, <1 1, 2	1, 1 1, 5	1, <1 1, 6	1, <1 1, 6	—	7, 1<1 1, 1	6, 1 2, 2	1, 3 4, 4 2, 5 1, 6	1, 3 4, 4 1, 5 2, 6

very active, intermediate compounds in the series, such as myristate with 14 carbon atoms and palmitate with 16 carbon atoms were much less so. This comparison between the activity of these acids has been repeated several times with identical results. It seems improbable that the effect was due to impurities in the fatty acids used; the effects recorded in Table 2 were obtained with acids which were 99–100 % pure, but if any of the effects observed with a particular compound were due to contamination by any of the others, the contaminating acid would need to have been present in a concentration of at least 10 % of the material. Nevertheless, it is conceivable that some quite different growth factor of high potency might have to be present in some of the acids.

The effect of fatty acids on the metabolism of organic acids

Hare *et al.* (1952) showed that strains of groups I, II, III and V were able to utilize certain organic acids, better growth and larger quantities of gas being produced in their presence than in control media not containing them. Experiments were therefore carried out to investigate whether sodium oleate at 355 μM had any effect on these reactions. There was no detectable effect on the behaviour of strains of groups I, II, V and VI towards these substances, but oleate stimulated the growth of both groups III and IV in pyruvate and more gas was produced by group III strains.

In other experiments, using 1 % (w/v) pyruvate and different concentrations of oleate it was found that in the presence of 355 μM or 35·5 μM-oleate, the pH of the medium fell from the initial 7·0 to 6·2–6·4 with group III, and to 5·6–6·0 in the case of group IV. With 3·5 μM-oleate or none, there was little, if any, fall in the pH value. Further investigations, using Simon & Piaux's (1924) qualitative method, showed that with both groups all the pyruvate had been utilized in the presence of the higher concentrations of fatty acids but little, if any, in the lower concentrations or in control tubes.

Effect of fatty acids on fermentation of carbohydrates

Hare *et al.* (1952) showed that strains of groups I and VI fermented a number of carbohydrates, whereas those of other groups had little or no activity. This inability to ferment may have been due in part to the fact that growth was very poor. Experiments were therefore carried out to determine whether the better growth obtainable in the presence of a fatty acid (e.g. oleate) might induce fermentation. The medium employed consisted of 2·0 % (w/v) peptone; 0·2 % (w/v) yeast extract; 0·1 % (w/v) sodium mercaptoacetate (thioglycollate); and 1 % glucose, maltose, fructose, sucrose or galactose. A control without carbohydrate was also included. Parallel series were set up, one without and the other with 355 μM-oleate. The tubes were incubated for 7 days and the volume of gas produced, the opacity of the culture and the pH value determined.

The general behaviour of groups I, II, V and VI was very little affected by the presence of oleate. Marked differences were observed with groups III and IV. With group III Hare *et al.* (1952) showed that in the medium they used (containing 5% peptone and 1% carbohydrate) these strains depressed the pH value slightly. A similar small decrease in pH value in media without oleate was obtained in these experiments (from pH 8·0 to 6·8–7·0) but in presence of oleate the pH values fell from an initial 8·0 to 5·4–5·6 in glucose, maltose, fructose or sucrose; growth was then very much better and large quantities of gas were produced. Group IV strains in the presence of oleate were similarly able to ferment fructose, the final pH value being 5·8–6·4 with 7 strains, but no gas was produced. In control media without oleate, these strains grew very badly and with no sign of fermentation.

In a second experiment using 1% fructose and different concentrations of oleate, it was found that with strains of group III as little as 3·55 μM-oleate sufficed to produce detectable fermentation whereas with strains of group IV 35·5 μM-oleate was necessary.

Growth on agar

Strains of groups III and IV grew very sparsely in broth without added fatty acid. But it was repeatedly observed that when the same broth was used to prepare agar plates, excellent growths were obtained after incubation for 48 hr. in a McIntosh & Fildes jar. Because relatively small quantities of fatty acids can stimulate growth, it seemed possible that substances of this nature were present as impurities in agar and were responsible for the better and more luxuriant growth on solid medium. Medium containing 1% (w/v) peptone, 0·2% (w/v) Difco yeast extract and 2·0% (w/v) Difco agar powder was made up and Norit (British Drug Houses Ltd.) added at different concentrations. The medium was sterilized by autoclaving. Representative strains, as 48 hr. broth cultures, were streaked over the plates which were incubated for 48 hr. in a McIntosh & Fildes jar. Strains of groups I, II and VI were indifferent, those of group V grew better on the agar containing charcoal and those of group III and IV were almost completely inhibited when Norit was present in a concentration as low as 0·012% (w/v).

To test whether toxic material had been extracted from the Norit, agar plates were prepared containing 0·2% (w/v) Norit. A ditch was then cut to one side of the plate and filled with agar containing the same concentration of Norit and 355 μM-oleate as well. As in the preceding experiment growth failed over most of the plates, but good growth was obtained over the ditch and in a zone of 8–100 mm. adjacent to it. It is, therefore, more probable that the failure of groups III and IV organisms to grow on agar containing Norit was due to the removal of fatty acids from the agar by the Norit and not to the presence of toxic material derived from the Norit.

Microscopic appearances of cocci in cultures with and without oleate

Microscopical examination of the sediment in tubes containing broth with different amounts of fatty acid showed that the presence or absence of the fatty acid made no difference to the arrangement or sizes of the cocci or groups I, II, V and VI; but with organisms of groups III and IV there was a definite correlation between size of cocci and fatty acid concentration. An experiment was then set up in which different concentrations of oleate were added to broth medium with and without 1% fructose. They were inoculated with representative strains of groups III and IV and incubated. Films were made of the deposit in the tubes after incubation for 24, 48 and 144 hr. The films were fixed by heat, cleared with acid ethanol and stained with carbol-fuchsin (0·1%) and examined; individual cocci were measured with an eyepiece micrometer.

The appearances after 24, 48 and 144 hr. incubation were similar; in the account given below, those seen after 48 hr. are described. Group III strains multiplied in the form of diplococci with the contiguous sides slightly flattened, but the size of the individual cocci varied with the concentration of oleate. In 355 μM-oleate they varied from 0·6 to 0·8 μ. diam. with a few of 1·0 μ.; in 35·5 μM and 3·55 μM-oleate, they were 1·0 to 1·2 μ. diam.; without oleate the cocci were as large as 1·2 to 1·6 μ. diam. The presence of fructose made no detectable difference to the sizes or arrangement of the organisms despite the fact that the growth was heavier.

On the whole, a similar picture was observed with group IV strains, the principal difference being that in absence of oleate or with only 0·355 μM or 3·55 μM-oleate, there were many poorly stained ghosts, although some of them had quadrants or crescents which were well stained. There was about the same number of well-stained large cocci (1·4 to 1·6 μ. diam.) and very few cocci 1·0 μ. diam. With 35·5 μM-oleate there was a range of sizes, some cocci being 1·0 to 1·2 μ. diam. but many were 0·5 to 0·8 μ. In 355 μM-oleate small cocci from 0·5 to 0·6 μ. diam. were everywhere in evidence; the larger forms were not easy to find, but some of 1·0 μ. diam. were present. There was much less tendency for the cocci to appear as diplococci than with group III organisms but, as with that group, fructose made no difference to the appearance of the individual organisms despite the fact that much better growth was obtained in its presence (see Pl. 1, figs. 1, 2, 3, 4, 5, 6). Thus, the diameter of group III and group IV cocci varied from 0·5 μ. to 1·6 μ. according to the fatty acid content of the medium.

The general appearance of the large cocci in films from cultures in which there was no oleate or only traces of it suggests that they were starvation cells, able to enlarge but incapable of carrying through the process of division to completion; preparations in which the cell wall was stained with methyl violet after mordanting with tannic acid certainly showed the presence of transverse septa in some cells (see Pl. 1, figs, 7, 8, 9). There was no evidence that all the large forms were multicellular. These large forms are comparable to those seen when some species of bacteria are grown in Mg-deficient media (Webb, 1948), in sub-lethal concentrations of dyes, such as methyl-violet (Ainley-Walker & Murray, 1904), or in penicillin (Gardner, 1940).

DISCUSSION

From these experiments it seems obvious that in the presence of certain fatty acids, e.g. oleate, the amount of growth, ability to attack carbohydrates or organic acids, formation of gas and morphology of the cocci of groups III and

IV, may be considerably modified. Although fatty acids have been known for some time to inhibit the growth of some species of organism, they have also been recognized as growth factors for other species; Fleming (1909), for instance, observed this in the acne bacillus, Dubos (1947) with *Mycobacterium tuberculosis* and Thoma & Peterson (1950) with certain clostridia. Williams & Fieger (1946) were unable to detect any connexion between the structure of a fatty acid and its activity as a growth-enhancing agent for *Lactobacillus casei* and considered that its activity was due principally to its physical properties. On the other hand, Boughton & Pollock (1952, 1953) observed that the length of chain and the position of the double bond in the fatty acid molecule were of importance in the promotion of the growth of a diphtheroid organism; they obtained data which suggested that the fatty acid formed a constituent part of the cell. Furthermore, unsaturated acids were more effective than saturated acids. Boughton & Pollock suggested that the growth-enhancing activities of a saturated fatty acid was due to its ability to overcome the toxicity of excessive amounts of unsaturated fatty acid present in the medium.

The data in this paper are insufficient to show the way in which fatty acids increase so very considerably the growth and metabolic activities of certain of the anaerobic cocci. Although the most effective acids are those which are surface active, it seems improbable that their function is to break up masses of cells, for there is no greater degree of bunching or agglomeration of the cells when oleate is absent or deficient than when it is present in sufficient concentration. On the other hand, in media deficient in fatty acid, individual cells are very large indeed and although many of them possess septa, the two components are each larger than one cell which has grown in an adequate concentration of oleate. This appearance suggests that in such cells there has been elaboration of sufficient foodstuffs and the necessary enzymes for the first phases of division, but that the later stages cannot be carried through. Since somewhat similar appearances are seen in cells subjected to sub-lethal concentrations of antibiotics or dyes, it seems probable that lack of an essential metabolite is responsible. On the evidence at present available it is impossible to say whether this metabolite is the fatty acid itself or whether the latter can so alter the permeability of the cell as to permit better absorption.

If fatty acids are of importance in the metabolism of the anaerobic cocci, it follows that the concentration of substances of this nature in the media used for their growth becomes a matter of considerable importance. There seems little doubt that the basal liquid medium we used was deficient in such substances; when incorporated in agar the growth-supporting qualities of the medium became so much better as to suggest that this was due to fatty acids present in the agar as impurities. Ley & Mueller (1946) showed that material having the chemical characteristics of a fatty acid can be extracted from agar and Pollock (1947, 1948) has also, to some extent, confirmed the presence of such substances in agar. Pollock found that the addition of charcoal to agar inhibited the growth of a corynebacterium which required a fatty acid, while the growth of a strain of *Haemophilus pertussis* which was inhibited by the presence of fatty acids, only grew on agar to which charcoal had been added. In our experiments, the presence of charcoal prevented growth of strains of groups III and IV on agar medium, but the same medium supported growth when oleate was allowed to diffuse into it. Although this suggests that oleate is a growth requirement for these strains, it does not preclude the possibility that the oleate had displaced some other metabolite from the surfaces of the carbon particles. Attempts to remove the charcoal in order to settle this point were only partially successful, but there were no indications that if its complete removal had been achieved, the results would have been different.

It is possible that very small quantities of fatty acid may be present in the peptone used, since Hare *et al.* (1952) obtained better growth of strains of groups III and IV in the presence of 5% (w/v) peptone than in 2% (w/v), and found that, although the diameter of the strains of group IV varied from 0·4 to 0·8 μ., there were no large forms of the kind regularly produced in 2% (w/v) peptone without added fatty acid. The large forms were observed, however, with group III strains in the 5% (w/v) peptone media. In view of the evident importance of fatty acid concentration in modifying the growth, fermentation reactions and morphology of anaerobic cocci belonging to groups III and IV, it is obvious that unless the media used are carefully standardized, these criteria are worthless for purposes of classification.

Our thanks are due to Messrs Lever Bros. for the gift of the samples of purified fatty acids, to Mr Albert Clark for the photographs, and to Mrs D. N. Twort and Mr R. Priddy for technical assistance. One of us (P. W.) was in receipt of a grant from the Leonard S. Dudgeon and Michael and Sidney Herbert Funds, St Thomas's Hospital Medical School.

REFERENCES

Ainley-Walker, E. W. & Murray, W. (1904). The effect of certain dyes upon the cultural characters of the *Bacillus typhosus* and some other micro-organisms. *Brit. med. J.* **2**, 16.

Boughton, B. W. & Pollock, M. R. (1952). Long chain unsaturated fatty acids as essential bacterial growth factors. *Biochem. J.* **50**, xxxi.

Boughton, B. W. & Pollock, M. R. (1953). Long chain unsaturated fatty acids as essential bacterial growth factors: further studies with *Corynebacterium* Q. *Biochem. J.* **53**, 261.

Brown, K. C. & Kirwan, E. W. O. G. (1914–15). Standardization of bacterial suspensions by opacity. *Indian J. med. Res.* **2**, 763.

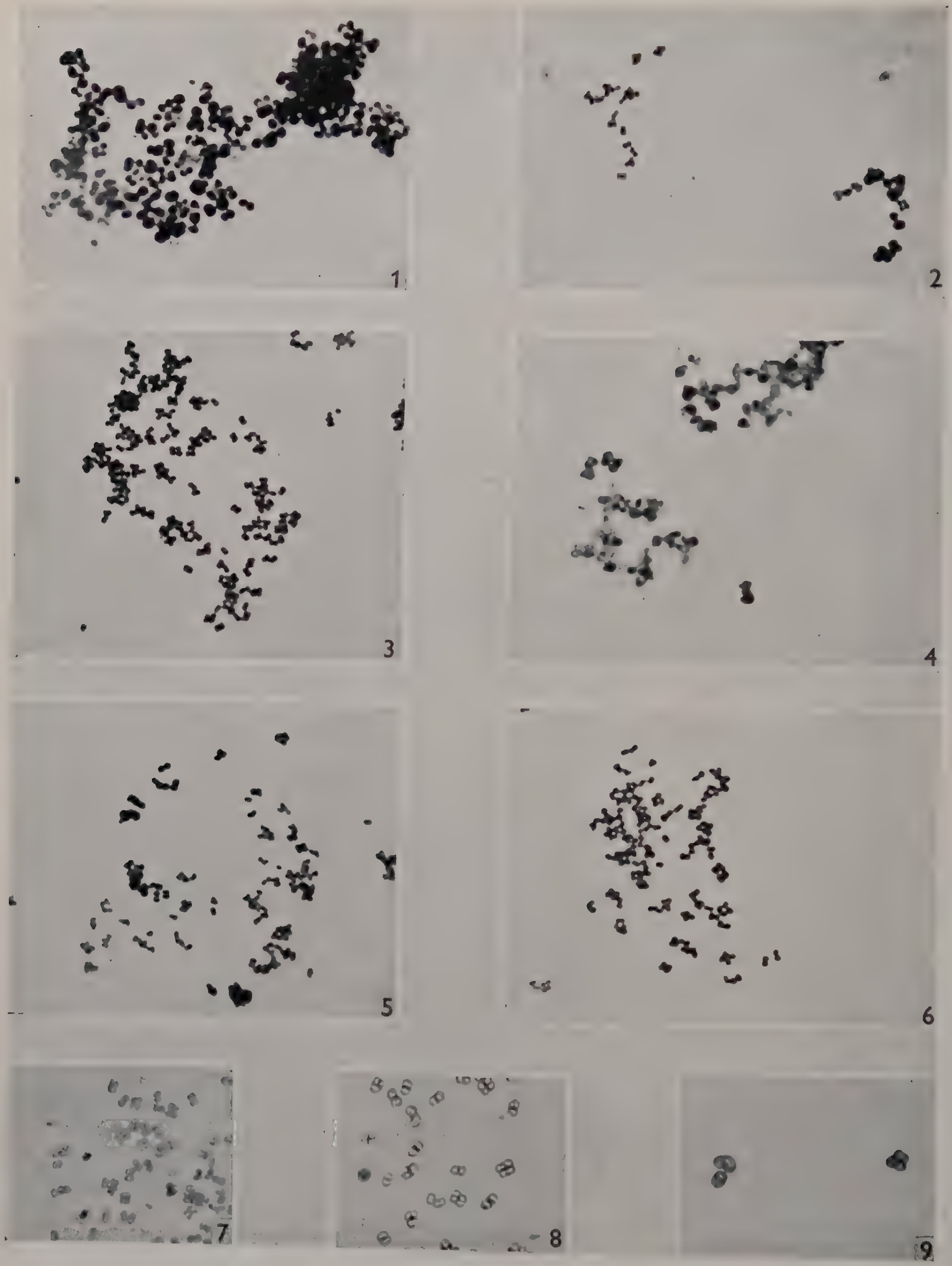
Dubos, R. J. (1947). The effect of lipoids and serum albumen on bacterial growth. *J. exp. Med.* **85**, 9.

Fleming, A. (1909). On the aetiology of acne vulgaris and its treatment by vaccines. *Lancet*, i, 1035.

Gardner, A. D. (1940). Morphological effects of penicillin on bacteria. *Nature, Lond.* **146**, 837.

Hare, R., Wildy, P., Billett, F. S. & Twort, D. N. (1952). The anaerobic cocci: gas formation, fermentation reactions, sensitivity to antibiotics and sulphonamides. Classification. *J. Hyg., Camb.* **50**, 295.

Klieneberger, E. (1934). The colonial development of the organisms of pleuropneumonia and agalactia on serum agar and variations of the morphology under different conditions of growth. *J. Path. Bact.* **39**, 409.



P. Wildy & R. Hare—Effect of fatty acids on anaerobic cocci. Plate 1

Ley, H. L. & Mueller, J. H. (1946). On the isolation from agar of an inhibitor for *Neisseria gonorrhoeae*. *J. Bact.* **52**, 453.

Pollock, M. R. (1947). The growth of *H. pertussis* on media without blood. *Brit. J. exp. Path.* **28**, 295.

Pollock, M. R. (1948). A case of bacterial symbiosis based on the combined growth-stimulating and growth-inhibitory properties of long-chain unsaturated fatty acids. *J. gen. Microbiol.* **2**, xxiii.

Robinow, C. (1943–44). Cytological observations on *Bact. coli*, *Proteus vulgaris* and various aerobic spore forming bacteria with special reference to the nuclear structures. *J. Hyg., Camb.* **43**, 413.

Simon, L. J. & Piaux, L. (1924). Sur la caractérisation et le dosage de petits quantités d'acide pyruvique. *Bull. Soc. Chim. biol. Paris*, **6**, 477.

Thoma, R. W. & Peterson, W. H. (1950). Biotin and arginine replacements in the nutrition of *Clostridium sporogenes*. *J. Bact.* **60**, 39.

Webb, M. (1948). The influence of magnesium on cell division. 1. The growth of *Clostridium welchii* in complex media deficient in magnesium. *J. gen. Microbiol.* **2**, 275.

Williams, V. R. & Fieger, E. A. (1946). Oleic acid as a growth stimulant for *Lactobacillus casei*. *J. biol. Chem.* **166**, 335.

Wright, H. D. (1934). A substance in cotton wool inhibitory to the growth of the pneumococcus. *J. Path. Bact.* **38**, 499.

EXPLANATION OF PLATE

(Magnification ×1080)

Figs. 1–6. Group IV strain. Sediment from 2% peptone broth cultures, fixed by heat, cleared with acid ethanol and stained with carbolfuchsin 0·1%.

Fig. 1. 48 hr. incubation. No oleate.
Fig. 2. 48 hr. incubation. 35 μM-oleate.
Fig. 3. 48 hr. incubation. 355 μM-oleate.
Fig. 4. 144 hr. incubation. No oleate.
Fig. 5. 144 hr. incubation. 35 μM-oleate.
Fig. 6. 144 hr. incubation. 355 μM-oleate.

Figs. 7–9. Group IV strain. Impression preparations from agar plates containing 0·2% (w/v) Norit. Ditch cut to one side and filled with agar containing 0·2% (w/v) Norit and 355 μM-oleate. Inoculated by streak across and incubated anaerobically for 48 hr. Fixed with Bouin's fluid (Klieneberger, 1934), mordanted for 2 hr. with 10% tannic acid and stained with 0·2% crystal violet (Robinow, 1943–44). Mounted in balsam.

Fig. 7. Cocci over the ditch.
Fig. 8. Cocci from area 5 mm. from ditch.
Fig. 9. Cocci from area 10–15 mm. from ditch.

(*Received* 9 *March* 1953)

Manten, A. & Rowley, D. (1953). *J. gen. Microbiol.* 9, 226–233.

Genetic Analysis of Valine Inhibition in the K12 Strain of *Bacterium coli*

By A. MANTEN
National Institute of Public Health, Utrecht, Holland
and D. ROWLEY
The Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, Paddington, London, W. 2

SUMMARY: Development of resistance to valine by the K12 strain of *Bacterium coli* (*Escherichia coli*) has been found to occur by a one-step process leading to full resistance in every case. The genetic factors controlling this valine resistance have been studied by recombinant analyses. The results indicate a very close linkage between valine resistance and leucine independence. These substances have previously been shown to bear special relationship to each other with respect to the K12 strain.

It has been observed recently that various bacteria which are capable of growth on media containing ammonia as the sole source of nitrogen are unable to grow when minute amounts of certain specific amino-acids are added to the medium. One of the most remarkable examples is that of the well known K12 strain of *Bacterium coli* (*Escherichia coli*) used by Lederberg & Tatum (1946) for their genetic studies. This strain is completely inhibited by less than 1 μg. DL-valine/ml. of the medium (Tatum, 1949; Rowley, 1953). All other amino-acids (except norleucine) appear to be inactive against this strain. The inhibition due to DL-valine can be prevented by the simultaneous presence of even smaller concentrations of leucine or isoleucine. So far as is known little genetic study has been made with these inhibitions and it seems desirable to ascertain to what extent this phenomenon is genetically controlled.

Various nutritionally exacting mutant strains derived from K12 (see Table 1) were, with one exception, found to be equally susceptible to inhibition by valine as was the parent strain. Valine-resistant strains were developed from these mutants by seeding heavily onto valine-containing plates. A series of crosses between valine-resistant and valine-sensitive mutants were then made and the nutritionally non-exacting (prototrophic) colonies developing from these recombinations were examined for their response to valine. Further crossings were seeded onto minimal medium containing thiamine or threonine to investigate possible linkage between these factors and valine resistance.

MATERIALS AND METHODS

The minimal medium used was that of Davis & Mingioli (1950). The mutant strains derived from K12 had requirements as shown in Table 1. The amino-acids used were all DL racemates as supplied by British Drug Houses Ltd. Sensitivity to valine was determined by streaking a bacterial suspension in sterile water up to ditches containing 100 μg. valine/ml. cut in minimal medium plates to which supplements had been added when required.

Table 1. *Sensitivity to valine and nutritional requirements of mutants derived from* K12

Strain	Requirement	Sensitivity to valine
K12	None	S
W677	Threonine, leucine, thiamine (TLB_1-)	R
58–161	Biotin, methionine (B.M. –)	S
J4–5	Tryptophan, histidine	S
J6–2	Tryptophan, histidine, proline	S
J5–3	Proline, methionine (P.M. –)	S
J5–10	Proline, histidine (P.H. –)	S

All supplements added at 20 μg./ml. except biotin which was 1 μg./ml.

RESULTS

Acquired resistance to valine

Of the mutant strains used only W677 appeared to be naturally resistant. The wild type (K12) and the derived mutants 58–161, J4–5 and J5–10 were used in further experiments as they gave sharply defined zones of inhibition with valine.

To determine the rate at which resistant cells develop, minimal medium plates containing 1, 2, 4, 10, 100 and 1000 μg. DL-valine/ml. were seeded with 3×10^8 cells of K12 and incubated at 37° for 2 days. It can be seen from Table 2 that approximately the same number of resistant cells arose with each concentration of valine.

Table 2. *Number of valine-resistant cells developing at varying valine concentrations*

Concentration of DL-valine (μg./ml.)	No. of cells inoculated	No. of colonies developing	Ratio; res./sens.
1	3×10^8	184	$6{\cdot}1:10^7$
2	3×10^8	196	$6{\cdot}5:10^7$
4	3×10^8	143	$4{\cdot}8:10^7$
10	3×10^8	167	$5{\cdot}6:10^7$
100	3×10^8	134	$4{\cdot}4:10^7$
1000	3×10^8	115	$3{\cdot}8:10^7$

When the resistant colonies which developed at each valine concentration were subsequently examined for sensitivity to valine, it was found that they were all resistant to 1000 μg. valine/ml. This rather surprising result is quite different from those which occur with penicillin or even streptomycin and it seems that resistance to valine develops as a one-step mutation, giving rise only to mutants of the completely resistant variety. As the development of resistance to penicillin has been described as 'obligatory multistep' and that to streptomycin 'faculative multistep' (Hsie & Bryson, 1950; Bryson & Szybalski, 1952) development of resistance to valine may be designated as belonging to an 'obligatory one-step pattern'. Valine resistance, once acquired, was maintained through many subcultures on media with and without valine. With the four valine-resistant strains used in these experiments no reversion to sensitivity could be detected after fifteen transfers on minimal medium or nutrient agar slopes.

Genetics of valine resistance

In an attempt to elucidate the genetics of valine resistance, crossing experiments were performed with the four K12 mutants 58–161, J4–5, J5–10 and W677. Of these mutants only the last is naturally resistant to valine, but resistant mutants of the first three strains were selected and are designated with the superscript '^{R}'.

Strains were grown overnight at 37° in liquid minimal medium supplemented with the appropriate amino-acids and vitamins as required (Table 1). Valine-resistant strains were cultivated in the presence of 1000 μg. DL-valine/ml. to ensure the growth of resistant cells only.

The incidence of genetic recombination was detected by applying Tatum & Lederberg's (1947) technique whereby the two cultures to be crossed are washed, mixed and seeded onto unsupplemented minimal medium agar. Minimal medium plates dried at 37° for 1 hr. were flooded with 2 ml. of the mixed bacterial suspension, thus distributing the bacteria evenly over the plates, and then dried while tilted at an angle from the horizontal. Controls to detect reverse mutation to prototrophy were carried out by seeding the separate mutants heavily on to minimal medium. The frequency of reverse mutation was always found to be negligible. Plates were then incubated for 2 or 3 days at 37°. The following crosses were attempted:

(1) 58–161^{R} × J4–5.
(2) 58–161^{R} × J5–10.
(3) 58–161^{R} × W677.
(4) 58–161^{R} × J4–5^{R}.
(5) 58–161^{R} × J5–10^{R}.
(6) 58–161 × J4–5^{R}.
(7) 58–161 × J5–10^{R}.
(8) 58–161 × W677.
(9) J4–5^{R} × J5–10.
(10) J4–5 × J5–10^{R}.

Of these crossings only nos. (2), (3) and (8) yielded sufficiently large numbers of prototrophic colonies and experiments with the other crossings were abandoned.

In one series of experiments in which approximately 10^9 cells of each of the two mutants were plated on minimal medium it was found that 58–161 × W677 and 58–161^{R} × W677 gave about 200 prototrophic colonies whilst 58–161^{R} × J5–10 yielded 30 prototrophic colonies per plate. The control plates never gave more than four colonies (usually 0–2) with the same inoculum. The apparent infertility of the other crosses is probably to be explained by the recent work of Hayes (1953) in which he has shown that K12 strains can exist in two mating types F+ and F−. It is probable that in many of the above crosses two F+ types were used.

Colonies picked from the recombination plates were examined for valine sensitivity. When practicable, all the colonies on the plates were examined but where large numbers of colonies developed, all the colonies in sectors of the plates were examined. This examination was done by suspending the colony in sterile water and streaking a loopful of suspension up to a ditch containing 100 μg. DL-valine/ml. cut in a minimal medium plate. Striking differences were observed in the proportions of valine-resistant prototrophs derived from the various crossings.

The cross of 58–161^R × J 5–10 showed a clear-cut segregation into completely resistant and completely sensitive colonies (Pl. 1, fig. 1). Of a total of 110 colonies tested, 64 were sensitive and 46 were fully resistant. These figures suggest that in the progeny of the cross there may be a slight preference in favour of valine-sensitive prototrophs. This may be due to factor linkage, as the same phenomenon has been encountered in the segregation of various factors in other crosses between K12 mutants (Lederberg, 1947).

A closer analysis of the location of the factor for valine resistance with respect to other factors on the bacterial chromosome was attempted. For these experiments the well-marked mutants 58–161 (B^-, M^-, T^+, L^+, $B_1{}^+$) and W 677 (B^+, M^+, T^-, L^-, $B_1{}^-$) were used. The symbols B^-, M^-, etc., indicate requirement for biotin, methionine, etc., whereas B^+, M^+, etc., indicate that the organism is independent of these factors.

The W 677 mutant cannot be grown in the absence of leucine from the medium. As leucine neutralizes the inhibitory effect of valine (Rowley, 1953) the question arises whether the valine resistance in W 677 lies solely in the presence of leucine in its medium, in other words that valine resistance in W 677 is merely phenotypical. The alternative is that the resistance to valine of W 677 has an endogenous basis, i.e. it is genotypical and can then be compared with induced valine-resistant strains such as the 58–161^R strain. A definite answer to this question can only be given by a crossing experiment. Should W 677 prove to be genotypically sensitive, then on crossing with the valine-sensitive 58–161 it would behave as if it were a cross of the type Val^S × Val^S and all the progeny would inevitably be valine sensitive. If, however, valine resistance of W 677 were genotypical, then a crossing with the sensitive 58–161 would be of the type Val^S × Val^R and consequently a mixture of sensitive and resistant progeny would be expected. The experimental crossing yielded colonies (191) all of which were sensitive to valine (Pl. 1, fig. 2). This is consistent with W 677 being genotypically sensitive to valine. From this conclusion it was to be expected that the cross of W 677 × 58–161^R would yield a mixture of sensitive and resistant colonies as was the case with J 5–10 × 58–161^R. However, the progeny from crossing W 677 × 58–161^R comprised 218 colonies all of which were resistant to 100 μg. valine/ml. (Pl. 1, fig. 3).

The problem arises as to how we can reconcile the results of crossing 58–161^R × J 5–10 with those from crossing 58–161^R × W 677. The most likely explanation would seem to be that there is a strong linkage of valine resistance to one or more of the factors responsible for prototrophy. In the cross W 677 × 58–161^R the factors implicated are (B^+, M^+, T^+, L^+, $B_1{}^+$) but only the latter three need be considered since B^+ M^+ play a role in the cross J 5–10 × 58–161^R where no linkage between valine resistance and factors for prototrophy (P^+, H^+, B^+, M^+) were found. It has been shown that the factor for thiamine independence, $B_1{}^+$, and the two factors for threonine and leucine independence, T^+, L^+, are located on the chromosome in distal positions (Lederberg, 1947; Cavalli, 1952). It seems likely therefore that the locus for valine resistance is situated either near to that for thiamine independence or in the vicinity of the threonine and leucine loci.

To decide between these alternatives W677 × 58–161^R were crossed on minimal medium supplemented with 20 μg. thiamine/ml.; thiamine is not antagonistic to valine inhibition. Crossing on this medium does not select for thiamine independence (B_1^+), and therefore B_1^- recombinants can also develop. Considering the exceptionally high degree of linkage to be accounted for, in the hypothetical case of linkage of Val^R to B_1^+, or the equivalent of Val^S to B_1^-, we expect all, or at least nearly all, B_1^- recombinants to be sensitive to valine. Since we expect that among the B_1^+ and B_1^- recombinants the B_1^- class will prevail (Lederberg, 1947), linkage of Val^S to B_1^- would be apparent if the majority of colonies developing on thiamine supplemented plates were sensitive. If this should not be found, the alternative of linkage of Val^R to T^+ and L^+ would hold true by elimination.

In the experiment colonies were picked and assayed for valine resistance (ditch plate technique) and thiamine requirement (presence or absence of growth on minimal medium). From a total of 62 colonies, 57 were thiamine dependent (as anticipated), yet not one valine-sensitive colony was found. This eliminates the possibility of linkage between Val^S and B_1^-, or Val^R and B_1^+, and leaves us with the alternative conclusion that Val^R must be linked with the T^+ or L^+ factors.

Finally, to decide whether Val^R is linked with the T or the L factors 58–161^R × W677 were seeded onto plates supplemented with threonine. Out of a total of 147 recombinant colonies (all threonine independent) only 7, or 5%, were of normal sensitivity to valine. The very high yield (95%) of Val^R colonies in this experiment suggests that the factor for valine resistance is situated in the close proximity of the L locus. A more definite conclusion as to the exact relation of Val^R with respect to L^+ or T^+ cannot be given, since the position of T^+ relative to L^+ is still undetermined (Lederberg, Lederberg, Zinder & Lively, 1951).

DISCUSSION

The development of resistance to valine in the K12 strain of *Bact. coli* may be unique in that it appears to occur by one step only. So far as we are aware this obligatory one-step development of resistance has only been described previously in the case of *Mycobacterium tuberculosis* and isonicotinic acid hydrazide (Middlebrook, 1952). In that instance, however, only a 15-fold range of concentration was examined, compared with a 1000-fold range in the present work. The crossing experiments fully confirm this one-step development of resistance since a clear-cut segregation of the progeny into fully resistant and fully sensitive strains was found.

Resistance to valine was found to be permanent, in common with resistance to some antibiotics, e.g. streptomycin. Attempts were made to isolate valine-dependent strains by similar techniques to those used by Miller & Bohnhoff

(1947) in isolating streptomycin-dependent variants of meningococci. Minimal medium plates containing 1000 μg. DL-valine/ml. were seeded with K12 and incubated for several days. Each day the newly developed colonies were examined for valine dependence by streaking on minimal medium ditch plates with a solution containing 100 μg. DL-valine/ml. in the ditch. Occasional valine-dependent strains were met but they were very unstable, quickly reverting to normal valine resistance. These dependent strains could in no way be compared with the stable amino-acid dependent mutants of K12 which have been isolated by other well-known methods (Lederberg, 1947).

In the recombination data summarized in Table 3, Exps. 2 and 3 yield conflicting results. From Exp. 2 we deduce that W677 is genotypically sensitive and from Exp. 3 we may draw exactly the opposite conclusion. One of these crosses must be complicated by a very strong linkage to factors for prototrophy. It seems quite possible that either Val^{S} or Val^{R} might be linked to L^{+} T^{+} or B$_1^{+}$. However, since all the other K12 mutants above are valine sensitive, it seems likely that W677 is genotypically sensitive also. Furthermore experiment 5, in which 7 sensitive recombinant colonies developed, proves that W677 must be Val^{S}, otherwise the progeny of its cross with 58–161^{R} would always be Val^{R}.

Table 3. *Summary of recombination data*

Exp. no.	Cross	Supplements	Colonies developing Val^{R}	Colonies developing Val^{S}
1	58–161^{R} × J5–10	None	46	64
2	58–161 × W677	None	0	191
3	58–161^{R} × W677	None	218	0
4	58–161^{R} × W677	B$_1$	62	0
5	58–161^{R} × W677	T	140	7

Control plates seeded with equivalent concentrations of each parent alone never showed more than four colonies.

A new approach to linkage data in K12 is necessary as a result of the recent work of Hayes (1953), and of Cavalli, Lederberg & Lederberg (1953). These authors have shown quite clearly the occurrence of two mating types designated F+ and F−; crosses of F− × F− are sterile and maximum fertility is shown by F+ × F− crosses. In the opinion of Hayes, the F− parent plays a more passive acceptor role, and in order to form a prototrophic recombinant from auxotrophic F+ and F− strains, the donor F+ strain must transfer the prototrophic loci which the F− form lacks. It is apparent, however, that only a part of the F+ chromosome is transferred to the F− cell. Exactly what this part is depends of course on the conditions of selection used in any experiment. Thus in a cross such as we have used, between 58–161/F+ and W677/F−, seeded on to minimal medium, there is selection for the TL+ part of the F+ chromosome and also for the B$_1$+ part. Any unselected markers in these portions of the F+ chromosome will tend to be carried over simultaneously. The degree to which they are carried over will depend on their proximity to the selected markers.

On the basis of these new concepts the data above can be re-interpreted as follows. Exps. 2 and 3 of Table 3, in both of which there is selection for TL+ and B_1+, show that Val^S and Val^R are associated either with the TL region or with the B_1 region. Exp. 4, however, eliminates selection for B_1+, nevertheless all the recombinants have the same Val^R as the F+ parent, showing that the factor controlling valine resistance is located in the TL region and presumably very close to the TL loci. Exp. 5 confirms this and since there is some separation of the Val^R marker from the TL markers, one must conclude that Val^R is very close to, but certainly not identical with, the TL loci. The results from Exp. 1 are very probably due to an F+ × F+ cross. This has been shown by Hayes to behave qualitatively as an F+ × F− cross mixed with the same cross of reversed F polarity. Thus Exp. 1 would be expected to yield a mixture of progeny from the two crosses below:

(*a*) $Val^R.M-/F+ \times Val^S.P-H-/F-$ (selection for P+ H+),
(*b*) $Val^R.M-/F- \times Val^S.P-H-/F+$ (selection for M+).

Since neither of these crosses involves transfer of the TL region there will be no transfer of the associated Val factor and the prototrophs will have the same valine sensitivity as the F− parent. The result will be a mixture of valine-sensitive and valine-resistant prototrophs in the ratio of the efficiency of the two recombining systems.

The conclusions, as to the position of Val^R in the TL region, remain valid. The reasoning, by which this conclusion was reached earlier in the discussion, must now be considered as invalid and is only included in order to demonstrate, as Hayes has already done, the difficulties in postulating linkages without carefully considering the F+ polarity of the cross. The crossing experiments show that valine resistance is controlled by one gene which is closely linked with the gene for leucine synthesis.

It is tempting to speculate that the highly specific reversal of valine inhibition of K12 by leucine may be a direct result of the close proximity of these two governing genes.

We are grateful to Sir Alexander Fleming for his continued encouragement and to Professor R. Cruickshank for helpful criticism of this paper. The mutants W677 and 58–161 were kindly supplied by Dr Forrest Fulton of the London School of Hygiene and Tropical Medicine, and we are indebted to Dr B. D. Davis of the U.S. Public Health Service, Tuberculosis Research Laboratory, New York for the other mutants. Finally, we wish to thank Dr W. Hayes of the Postgraduate Medical School, London, for his illuminating discussions on these problems. One author (A. M.) began this study during the tenure of a W.H.O. Followship at the Wright-Fleming Institute.

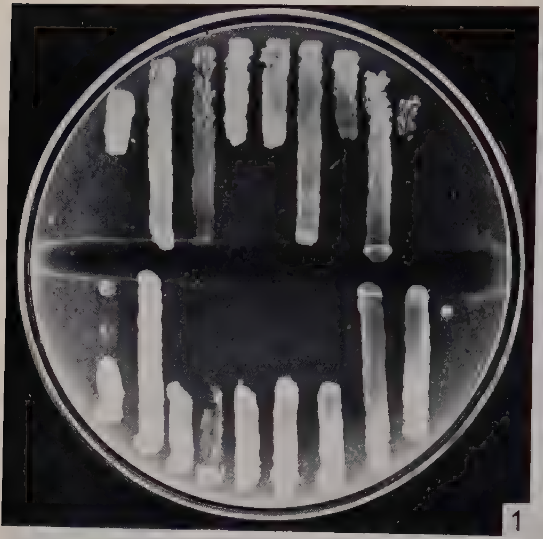
REFERENCES

Bryson, V. & Szybalski, W. (1952). Microbial selection. *Science*, **116**, 45.

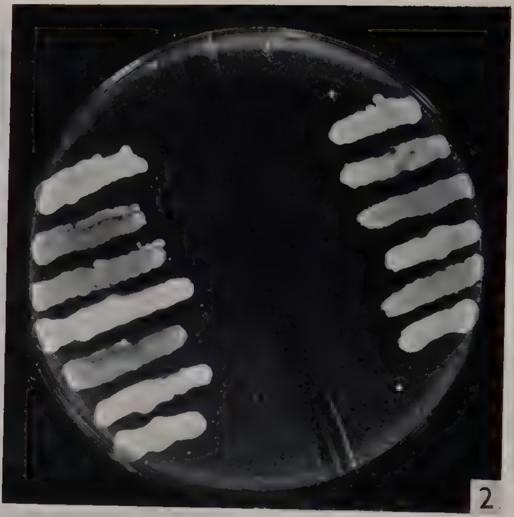
Cavalli, L. L. (1952). Genetic analysis of drug-resistance. *Bull. World Hlth Org.* **6**, 185.

Cavalli, L. L., Lederberg, J. & Lederberg, E. M. (1953). An infective factor controlling sex compatibility in *Bacterium coli*. *J. gen. Microbiol.* **8**, 89.

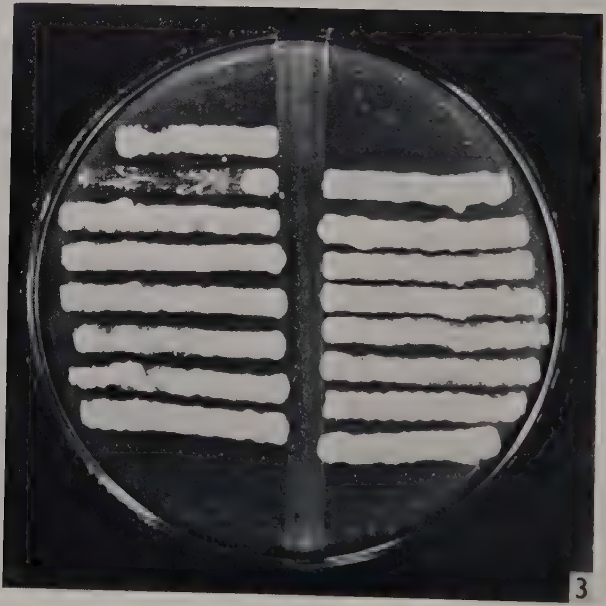
Davis, B. D. & Mingioli, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B12. *J. Bact.* **60**, 17.



1



2



3

A. MANTEN & D. ROWLEY—VALINE INHIBITION IN *BACT. COLI.* PLATE 1

Hayes, W. (1953). Observations on a transmissible agent determining sexual differentiation in *Bacterium coli*. *J. gen. Microbiol.* 8, 72.

Hsie, J. Y. & Bryson, V. (1950). Genetic studies in the development of resistance to neomycin and dihydrostreptomycin in *Mycobacterium ranae*. *Amer. Rev. Tuberc.* **62**, 286.

Lederberg, J. (1947). Gene recombination and linked segregations in *Escherichia coli*. *Genetics*, **32**, 505.

Lederberg, J., Lederberg, E. M., Zinder, N. D. & Lively, E. R. (1951). Recombination analysis of bacterial heredity. *Cold Spr. Harb. Sym. quant. Biol.* **16**, 413.

Lederberg, J. & Tatum, E. L. (1946). Gene recombinations in *Escherichia coli*. *Nature, Lond.* **158**, 558.

Middlebrook, G. (1952). Sterilization of tubercle bacilli by isonicotinic acid hydrazide and the incidence of variants resistant to the drug *in vitro*. *Amer. Rev. Tuberc.* **65**, 765.

Miller, C. P. & Bohnhoff, M. (1947). Two streptomycin resistant variants of meningococcus. *J. Bact.* **54**, 467.

Rowley, D. (1953). Inhibition of *Escherichia coli* strains by amino acids. *Nature, Lond.* **171**, 80.

Tatum, E. L. (1949). Amino-acid metabolism in mutant strains of microorganisms. *Fed. Proc.* **8**, 511.

Tatum, E. L. & Lederberg, J. (1947). Gene recombination in the bacterium *Escherichia coli*. *J. Bact.* **53**, 673.

EXPLANATION OF PLATE

Fig. 1. Prototrophic colonies from the cross 58–161^R × J 5–10 tested on minimal medium ditch plates. 100 μg. DL-valine/ml. in the ditch.

Fig. 2. Result of the cross 58–161 × W 677. Note all recombinants sensitive to 100 μg. DL-valine/ml.

Fig. 3. Result of the cross 58–161^R × W 677. Note all recombinants resistant to 100 μg. DL-valine/ml.

(*Received* 11 *March* 1953)

Briggs, M. (1953). *J. gen. Microbiol.* 9, 234–248

The Classification of Lactobacilli by means of Physiological Tests

By MARY BRIGGS

National Institute for Research in Dairying,
University of Reading

SUMMARY: Four hundred and fifty-two strains of lactobacilli, including representatives of all recognized species and 197 freshly isolated cultures, were studied. It was possible to classify 390 (86·3 %) of these into eight groups on the basis of the results of six physiological tests. The tests used were the production of gas from glucose and citrate, and of ammonia from arginine, growth at 15, 45 and 48°, heat survival at 60 and 65° and tolerance of 4, 6 and 8 % (w/v) sodium chloride.

The widespread habitat of organisms of the genus *Lactobacillus* and their importance in dairy, industrial and fermentation processes, have occasioned considerable interest for many years. Since the first reports in the literature at the end of the nineteenth century, many papers have been published dealing with the characteristics of the various species and their differentiation, but few workers have attempted a classification of all members of this genus. The existing groups have been incompletely described and characterized, so that it has been virtually impossible to identify strains of lactobacilli isolated from new sources. Such strains have frequently been given the status of new species and named accordingly. Although some workers have examined and correlated certain of these species, much confusion still exists.

Most of the earlier workers were interested in the organisms isolated from milk, the human intestine and faeces; differences in colonial and morphological appearances and in the fermentations of various sugars were the differential tests most commonly used. The distinction between *Lactobacillus acidophilus*, a homofermentative intestinal organism first described by Moro (1900), and various other lactic acid bacteria has been studied by many workers. Weiss & Rettger (1934) found many similarities between *L. acidophilus* and another intestinal organism, *L. bifidus*, isolated by Tissier (1900) from the faeces of breast-fed infants, but Orla-Jensen, S., Orla-Jensen, A. D. & Winther (1935–6) considered these two organisms to be different; recently Friedman & Black (1941) have supported this view. Various reports describe the identification of *L. bulgaricus*, first isolated by Grigoroff (1905) from fermented milk, but the evidence is still not convincing. Heinemann & Hefferan (1909) suggested that *L. bulgaricus* was a synonym for *L. acidophilus*, but Rahe (1918) and Kulp & Rettger (1924) maintained that these organisms could be separated on the basis of sugar fermentations. Kulp (1929), Curran, Rogers & Whittier (1933) and Sherman & Hodge (1940) reported distinct differences between the two organisms.

For many years the organisms found in dental caries were thought to belong to a distinct species, but Howe & Hatch (1917) suggested that these organisms were lactic acid bacteria similar to the Moro-Tissier group. However, McIntosh, James & Lazarus-Barlow (1922, 1924) considered them to be a separate group and named them *Bacillus acidophilus-odontolyticus*. Rosebury, Linton & Buchbinder (1929) and Hadley, Bunting & Delves (1930) found little difference between dental caries organisms and *L. acidophilus*, but Morishito (1929) separated them by using sugar fermentation reactions. Very few intensive studies of the differences between other homofermentative lactobacilli have been undertaken, although Sherwood (1939) reported that *L. casei* was morphologically different from *L. plantarum* and also produced more acid, and Burri & Kollman (1941) suggested that growth at 51° and 20° distinguished *L. lactis* from *L. helveticus*. The gas-producing species of lactobacilli include numerous variously named organisms, many of which are probably identical. The most important investigations of this group were made by Pederson (1938), who divided it into four sections on the basis of correlation between growth temperatures and sugar fermentations, and by Shimwell (1948), who used similar characteristics in his classification of the brewery lactobacilli, which include both homo- and heterofermentative organisms.

In his classic work, Orla-Jensen (1919) was the first to study the complete family Lactobacteriaceae. From the results of this and subsequent work, Orla-Jensen (1942, 1943) divided the catalase-negative rods (genus *Lactobacillus*) into three groups, the high- and low-temperature homofermentative groups which are named *Thermobacterium* and *Streptobacterium* respectively, and a heterofermentative group which he called *Betabacterium*. The organisms within these groups were mainly subdivided by means of the optical rotation of the lactic acid produced, sugar fermentations and the quantity of acid formed in milk. Although this classification was a great improvement on all earlier attempts, it was not completely satisfactory as a considerable number of intermediate and unclassifiable organisms was found.

The organisms of the genus *Lactobacillus* Beijerinck, included in the family Lactobacteriaceae Orla-Jensen, are described by Pederson (1948) (in *Bergey's Manual of Determinative Bacteriology*, 1948) as Gram-positive rods which ferment carbohydrates and polyalcohols either by homofermentation to lactic acid or by heterofermentation to lactic and acetic acids, alcohols and carbon dioxide. They are catalase-negative, microaerophilic, non-motile and do not produce spores; they do not reduce nitrate to nitrite, liquefy gelatin or grow on potato.

The classification of lactobacilli by Pederson in *Bergey's Manual* (1948) is largely based on Orla-Jensen's work, but the descriptions of the various organisms are supplemented by observations of other workers. The homofermentative group is subdivided by tests similar to those described by Orla-Jensen, and the heterofermentative species according to Pederson (1938); although this appears admirable in theory, it is found to have distinct practical limitations. For some species, such as *L. acidophilus* and *L. helveticus*, no differentiating characteristics are quoted, but perhaps the most serious criticism concerns the variability of many of the distinguishing reactions described. Sugar fermentations, for example, have been found unreliable in that they have frequently failed to give consistent and reproducible results, not only in the hands of different workers, but also with the same individual on different occasions (Weaver, 1932). The optimum temperature of growth for a given organism is a vague characteristic depending on many factors such as the medium used and the vigour of the particular culture. It is a characteristic difficult to determine accurately within a given range, yet *Bergey's Manual* suggests a subdivision of the heterofermentative lactobacilli based largely on the difference between optimum temperatures of 28–32° for one group and 35–40° for the other. The time and labour involved in the determination of the optical rotation of lactic acid is a serious drawback to the use of this test for purposes of differentiation.

The type species of the genus is *L. caucasicus* Beijerinck. This organism was isolated from kefir by Beijerinck (1889) and is of considerable historical interest, but its early description lacks detail and no worker recently has succeeded in isolating a similar organism from kefir. The lactobacillus now found in kefir is a heterofermentative organism named *Betabacterium caucasicum* by Orla-Jensen and confusion exists between this organism and the homofermentative type species, *L. caucasicus*. Tittsler, Geib & Rogosa (1947) suggested a system of classification based on a correlation of physiological and biochemical tests, but although the preliminary account of this work appeared promising, no details have yet been published. An account of a symposium on the lactic acid bacteria containing considerable information of a general nature has been published recently (Tittsler, Pederson, Snell, Hendlin & Niven, 1952). A complete review of the relevant literature is beyond the scope of this paper, but the few references quoted show that there is much conflicting, contradictory and unreliable evidence. Reference to the number of organisms included in Appendix I of the list in *Bergey's Manual* (pp. 361–4) will leave no doubt that further investigation of the genus *Lactobacillus* is necessary.

The object of the present work was to establish simple, reproducible and reliable tests for the differentiation and characterization of the lactobacilli. Examination of the literature indicated that much of the contradictory evidence reported might be traced to the use, in the same tests, of different media by various workers. As a preliminary to this work, a standard basal medium was evolved (Briggs, 1953) in which all strains of lactobacilli grew well, so that valid comparisons could be made.

METHODS

Cultures used

A collection of some 100 strains of lactobacilli has been maintained at this Institute for many years and these cultures were used during the preliminary trials of various physiological tests. It was found possible to group these organisms by means of six tests which are described below. Additional named strains were then obtained from many different collections so that further representatives of all groups could be tested similarly. Finally, strains of freshly isolated lactobacilli from different sources were tested to determine whether these also could be grouped in the same way. Before the strains were transferred to stock and subsequently tested, single colonies were picked, the resulting cultures tested for catalase production and a note was made of the colony forms, morphological appearances and Gram-reactions. A list of the 452 cultures included in the final collection with the total numbers of each 'species' or set of cultures and the appropriate code numbers is shown in Table 1.

Several representatives of certain named strains are included; these were obtained from different sources and all were tested to determine whether the effects of various methods of maintenance had affected their specific reactions.

Media

Storage. Stock cultures were stored at 4° in yeast glucose litmus milk plus chalk (Davis, 1935) and renewed every 3 months. A minimum of four transfers was made in yeast glucose litmus milk before the cultures were incubated in yeast glucose litmus milk plus chalk and replaced in the refrigerator.

Broth and agar media. The composition of the tomato glucose broth used throughout this work was : tomato juice, 10 ml.; Neopeptone (Difco), 1·5 g.; glucose, 2 g.; sodium chloride, 0·5 g.; Tween 80 (polyoxyethylene sorbitan monooleate), 0·1 ml.; Yeastrel, 0·6 g.; soluble starch, 0·05 g.; distilled water to 100 ml. The pH value was adjusted to 6·8 and the medium was autoclaved at 15 lb./sq.in. for 20 min. Further details of this medium have been given in an earlier paper (Briggs, 1953).

The tomato juice was prepared by adding 1 l. of distilled water to 12 lb. of tomatoes, mincing and then pulping in a metal press. The juice was filtered, neutralized to pH 7·0 and autoclaved at 120° for 15 min. Ten ml. of this concentrated juice were equivalent to 40 ml. of the tomato juice prepared as previously described (Briggs, 1953). Tomato glucose agar, prepared by the addition of 2 % agar to the above medium, was used for plating. The plates were incubated anaerobically in an atmosphere of 90 % hydrogen + 10 % (v/v) carbon dioxide.

All strains of lactobacilli grow well in the broth and in the agar media. Special media were necessary for the detection of gas production; tomato glucose broth was used, however, as the basal medium for all other physiological tests, in which growth was detected by observation of opacity, the uninoculated tomato glucose broth being translucent.

Preparation of inoculum

False negative results of any test may be obtained unless the organisms are growing vigorously before inoculation. Inocula were prepared from stock cultures which were subcultured into yeast glucose litmus milk for two transfers and then into tomato glucose broth for at least two further transfers. Some of the slower-growing organisms required three or four subcultures in broth before regaining their full vigour. An inoculum of two double standard

Table 1. *Named cultures or sources of lactobacilli and code numbers*

Code no.*	Name† or source	No. of cultures
A1–A22	*L. acidophilus*	22
AA1	*L. acidophil-aerogenes*	1
AH1–AH54A	Lactobacillus species from Gruyère cheese	53
AR1–AR5	*L. arabinosus*	5
B1–B23	*L. bulgaricus*	23
BC1, BC2	*L. buchneri*	2
BF1, BF3, BF4	Lactobacillus species from infant faeces	3
BR1–BR33	Lactobacillus species from Stilton cheese	32
C1–C30	*L. casei*	30
CC1	*L. caucasicus*	1
CD1–CD3	Lactobacillus species from chick duodena	3
CP1–CP3	Lactobacillus species from New Zealand cheese	3
CS1–CS8	Lactobacillus species from dried stomachs of calves	7
D1–D9	*L. delbrueckii*	9
E1–E4	*L. pentoaceticus*	4
F1–F16	*L. fermenti*	16
FG1	*L. frigidus*	1
G1, G2	*L. gayonii*	2
GF1, GF2	Lactobacillus species from guinea-pig faeces	2
GORD	Lactobacillus species from pasteurized milk	1
H1–H21	*L. helveticus*	20
HL1	*L. hilgardii*	1
J1, J2	*L. jugurt*	2
K1	Lactobacillus species from kefir	1
KR1	Lactobacillus species from sauerkraut	1
L1–L19	*L. lactis*	18
LE2–LE6	*L. leichmannii*	5
LC1	Lactobacillus species from fish silage	1
M1	*L. mannitopoeus*	1
M6NO, MBP, MM5, MP8, ME10	Lactobacillus species from calf abomasa	5
MF1	*L. malefermentans*	1
MR1–MR5B	Lactobacillus species from raw milk	7
N1–N7	Lactobacillus species from pig and chick caeca	7
O1–O13	*L. odontolyticus* and L. species of oral origin	13
P1–P31	*L. plantarum*	30
PF1–PF7	Lactobacillus species from pig faeces	7
PN1, PN2	*L. pentosus*	2
PR1–PR11	Lactobacillus species from *présure*	11
PS1	*L. parvus*	1
R1	*L. brassicae-fermentatae*	1
RF1	Lactobacillus species from rabbit faeces	1
RM1	Lactobacillus species from bovine rumen	1
S1–S6	*L. bifidus*	7
SL1–SL50	Lactobacillus species from silage	28
SM1–SM10	Lactobacillus species from Jugoslavian sour milk	9
St1–St3	Lactobacillus species from pig stomachs	3
SM1A, S38A, S40A, S41A	Lactobacillus species from discoloured meat	4
T1–T16	*L. pastorianus* and L. species from beer	15
TH1	*L. thermophilus*	1
U1	*L. rudensis*	1
W1	Lactobacillus species from silage	1
WL1–WL45	Lactobacillus species from Sweden	10
X1–X4	*L. brevis*	4
Y1, Y2	*L. lycopersici*	2
YOG1–YOG5	Lactobacillus species from yoghourt	4
Y48, Y48(1)	Lactobacillus species from Denmark	2
27, 126, 237, 504	Lactobacillus species from pig intestines	4

* No. allotted to cultures when received.

† Name of culture as received.

(4 mm.) loopfuls of a 24 hr. culture into 10 ml. medium was used for all strains, except that with a few slow-growing varieties the same volume of inoculum from a 48 hr. culture was necessary.

Incubation temperatures

The incubation temperatures of the various species were chosen mainly on the basis of experience gained here in past years. Practical experience with individual cultures and reference to the temperatures used by other workers also influenced the choice. Strains received as *L. lactis* and *L. thermophilus* were incubated at 45°, strains of *L. acidophilus*, *L. bulgaricus*, *L. delbrueckii*, *L. fermenti*, *L. helveticus* and *L. leichmannii* at 37°, and strains of *L. brevis*, *L. casei*, *L. pastorianus* and *L. plantarum* at 30°.

Physiological tests

Production of gas from glucose. During a study of the sugar fermentation reactions of a few heterofermentative lactobacilli, it was noticed that no gas was produced in Durham tubes and that a more reliable method for testing the production of gas from glucose was required. The medium and technique of Gibson & Abd-el-Malek (1945) was used successfully, cultures being incubated for 14 days.

Production of gas from citrate. A method for the determination of the production of gas from citrate was also described by Gibson & Abd-el-Malek (1945). All the heterofermentative strains grew well and some produced gas in the citrate medium so that it was not considered necessary to supplement it with glucose as suggested by Gibson & Abd-el-Malek. Cultures were incubated for 14 days.

Production of ammonia from arginine. Niven, Smiley & Sherman (1942) reported a method for detecting the production of ammonia from arginine by streptococci. A modification of this method, in which tomato glucose broth was used as the basal medium, proved satisfactory for lactobacilli: 0·3% (w/v) L-arginine hydrochloride was added to tomato glucose broth before autoclaving; the same medium without arginine served as the control. Nessler's solution was used to test for the presence of ammonia after 14 days' incubation. It was necessary to read results immediately after the addition of the Nessler's solution as a greenish colour developed on standing. Determination of arginine hydrolysis by streptococci of known reaction gave identical results using the original and modified techniques.

Heat survival tests. Preliminary survival tests of a few strains at 60° and 65° for varying periods of time appeared promising. Heat survival tests of all cultures for 30, 60 and 90 min. periods at these two temperatures were carried out in triplicate in a water-bath having an accuracy of ±0·1°. Tubes of 10 ml. of tomato glucose broth were heated to the required temperature in the bath, nine tubes were removed, rapidly inoculated with the standard inoculum of each culture under test and then replaced in the bath. After allowing 2 min. for the tubes to regain the temperature of the bath, three tubes were removed after 30 min., three after 60 min. and the remaining three after 90 min. The tubes were cooled rapidly and incubated for 7 days. Inoculated controls were prepared by heating tubes in the bath and cooling to room temperature before inoculation. Investigation of a few strains indicated that testing at 65° was unnecessary for cultures which did not survive 90 min. at 60°.

Growth temperatures. A survey of the growth temperatures of ninety-five strains originally maintained here indicated that growth at 15°, 45° and 48° could be used as differential tests. The accuracy of the water-bath used for the tests at 45° and 48° was ±0·1° and the maximum variation of the 15° water-bath was 0·5°. The standard inoculum was added to 10 ml. tomato glucose broth at the appropriate temperature. Cultures were incubated for 7 days at 45° and 48° and for 21 days at 15°.

Tolerance of sodium chloride. The growth of a few strains in broths containing 2, 3, 4, 5, 6, 7 or 8% (w/v) sodium chloride suggested that further investigation of the effects of 4, 6 and 8% concentrations would prove helpful. Sodium chloride was added to tomato glucose broth (which contains 0·5% NaCl) to give the total salt concentration required. A standard inoculum of the culture to be tested was added to tubes of each of the three salt concentrations under test and also to a control tube of tomato glucose broth. Growth was observed during 14 days' incubation.

RESULTS

Four hundred and fifty-two cultures of lactobacilli were studied and 390 (86·3%) of them were separated into eight groups on the basis of the results of the physiological tests. Forty-seven of the remaining sixty-two unclassified organisms were found to constitute three distinct sections; a fourth section accommodated the remaining fifteen organisms. The summarized reactions of the organisms of each group and section are shown in Table 2, with the exception of the miscellaneous strains in section 4. Certain tests have been omitted from the table, only those of direct importance in the separation of the groups being included. The remaining tests, which gave somewhat variable results, are mainly of confirmatory value.

Organisms of group I

Group I contained thirty-four strains including thirteen of the named *L. acidophilus* cultures (A1–A5, A12–A17, A20 and A22). Seven named strains of *L. bulgaricus* (B4, B7, B15, B16, B19, B20 and B23), one of *L. delbrueckii* (D2) and thirteen freshly-isolated strains (BF4, CS2, N2, N4, N5, O13, PF2, PF3, PF6, Y48 (1), 27, 126 and 237) possessed the characteristic reactions of group I. Twelve of the freshly isolated cultures were of intestinal origin whilst the thirteenth (O13) was from a human mouth. Only two strains (A13 and A15) produced gas from citrate and few organisms survived heating for 60 min. at 60°.

Organisms of group II

Only three named strains of *L. acidophilus* (A18, A19, A21) and one of *L. bulgaricus* (of which duplicate cultures, B2 and B3, were tested) were included amongst the sixteen organisms in group II. The remaining eleven cultures (CS6, SM10, St1, St3, St5, WL1, WL2, WL7, WL12, Y48, 504) were either unnamed or freshly isolated. No strains in this group produced gas from citrate and only B2 survived heating for 60 min. at 60°.

Table. 2. *Summary of biochemical and physiological reactions of lactobacilli of each group and section*

Group	Gas from glucose	NH_3 from arginine	Survivals at		Growth at			Tolerance of 4% NaCl	No. of strains in group
			60° for 90 min.	65° for 30 min.	15°	45°	48°		
I	−	−	−	−	−	+	−	−	34
II	−	−	−	−	−	−	−	−	16
III	−	−	+	$-\left(\frac{13}{20}\right)^*$	−	+	−	−	20
IV	−	−	−	−	+	+	+	+	25
V	−	$-\left(\frac{79}{82}\right)$	+	+	−	+	$+\left(\frac{81}{82}\right)$	$-\left(\frac{81}{82}\right)$	82
VI	−	$-\left(\frac{125}{127}\right)$	−	−	+	−	−	$+\left(\frac{116}{127}\right)$	127
VII	+	$+\left(\frac{44}{55}\right)$	−	−	$+\left(\frac{54}{55}\right)$	−	−	$+\left(\frac{31}{55}\right)$	55
VIII	+	+	$-\left(\frac{27}{31}\right)$	$-\left(\frac{29}{31}\right)$	$-\left(\frac{30}{31}\right)$	+	$-\left(\frac{26}{29}\right)$	$-\left(\frac{20}{31}\right)$	31
					Total number of strains grouped:				390
Unclassified organisms									
Section 1	−	−	$+\left(\frac{15}{24}\right)$	−	−	+	+	$-\left(\frac{22}{24}\right)$	24
Section 2	−	−	$-\left(\frac{10}{11}\right)$	−	−	+	−	+	11
Section 3	−	−	−	−	+	+	−	$+\left(\frac{10}{12}\right)$	12
Section 4			Miscellaneous reactions						15
					Number of strains classified in Sections:				62
					Total number of strains examined:				452

− = negative; + = positive.

* Where all strains did not react uniformly, the figures in parentheses show

$$\frac{\text{No. of strains giving the reaction specified}}{\text{No. of strains tested}}$$

Organisms of group III

Seven strains received as *L. bulgaricus* (B8, B12–B14, B18, B21, B22) as well as seven named strains of *L. helveticus*, *L. lactis*, and *L. delbrueckii* (H12, H13, H16, H21, L18, L19, D1) were included in group III. Four recently isolated strains (AH41B, PR5, PR8, PR10) were from Gruyère cheese and *présure* whilst the remaining two organisms (WL19, WL20) in this group were unnamed. The inability to produce gas from citrate was characteristic of all twenty strains.

Organisms of group IV

The twenty-five strains: A8–A10, B1, D4, D8, C10, C14, C16, C17, C20, C21, C25, H2, H3, H5–H8, H10, H11, H15, O5, O9 and YOG3 constituted group IV. Fifteen were strains received as *L. casei* or *L. helveticus*, nine of which (C14, C16, C17, H2, H5, H6, H7, H8, H15) are believed to be cultures of *L. casei* ATCC 7469. Cultures bearing this collection number were received both as *L. helveticus* and *L. casei* and it is of interest that they all showed similar reactions. Rogosa (1946) suggested that *L. delbrueckii* L.D. 5 should be renamed *L. casei*; two strains of L.D. 5 (D4 and D8) in our collection showed the typical reactions of group IV. Only three freshly isolated organisms were included in this group. Fourteen cultures produced gas from citrate and almost all grew in the presence of 6% sodium chloride; little resistance was shown to heating at 60° for 60 or 30 min.

Organisms of group V

The eighty-two cultures in this large group showed thermophilic properties; not only did they grow at 48°, but all survived heating to 65° for 30 min. and many survived 90 min. at this temperature. Twelve strains of *L. lactis* (L1, L3–L5, L7–L10, L13–L15, L17), five of *L. leichmannii* (LE2–LE6) and one of *L. thermophilus* (TH1) were included as well as A6, B6, B11, D9, H14, H19, WL39 and WL45. All cultures from Gruyère cheese except AH4, AH5, AH8B, AH18B, AH19B, AH41B and AH53B showed the typical reactions of group V. Other freshly isolated cultures were from pasteurized milk (GORD), *présure* (PR2, PR4, PR6, PR7, PR11) and dried stomachs of calves (CS7, CS8). Three strains (LE3, LE4, D9) produced ammonia from arginine, a reaction shown by very few homofermentative lactobacilli.

Bergey's Manual (1948) describes *L. leichmannii* as similar to *L. delbrueckii* but with a lower optimum temperature for growth; it was surprising to find that all strains received as *L. leichmannii* grew at 48°. Only three out of nine strains named *L. delbrueckii* grew at this temperature, one (D9) was classified in group V and two (D4 and D8) in group IV.

Strains A2 (group I) and A6 (group V) bear the same collection number (NCTC 1899). It is reasonable to suppose that the great differences between the reactions of these two strains may be due to an error in labelling, possibly long-standing, rather than to a mutation during maintenance. Strains AH10 and PR6 were somewhat atypical in that the former produced gas from citrate and the latter grew in the presence of 4% sodium chloride. The survival of L1 (*L. lactis* Dorner) at 65° clearly demanded its inclusion in this group despite its inability to grow at 48°; all other reactions of this organism were typical of the thermophilic lactobacilli (group V).

Organisms of group VI

Group VI, the largest, contained 127 organisms. Eighteen of these (C1–C9, C11–C13, C18, C22–C24, C26, C29) were received as *L. casei* and twenty-six (P1–P9, P11, P12, P14–P26, P30, P31) as *L. plantarum*. All strains named

L. arabinosus or *L. pentosus* as well as the three cultures (CP1–CP3) from New Zealand cheese were in this group. D3, D5, H1, H4, H9, L18 and five oral strains (O1, O3, O6, O7, O11) also possessed the typical reactions of the group. Five cultures (E3, F5, T7, T8, X3) which, when received, were stated to be heterofermentative, and fifty-five freshly isolated strains from Stilton cheese, silage, raw and sour milk, and fish silage were also classified in group VI.

Few of these strains survived heating at 60° for even 30 min.; growth was variable in 4 and 6 % sodium chloride, and ten strains grew in the presence of 8 % salt. Gibson & Abd-el-Malek (1945) observed that one strain of *L. casei* produced gas from citrate, whereas two strains of *L. plantarum* did not. They suggested that examination of large numbers of these strains might provide a means for differentiating *L. casei* from *L. plantarum*. The present work has not confirmed this distinction.

Organisms of group VII

The fifty-five heterofermentative lactobacilli in group VII include many variously named cultures: *L. brevis*, *L. buchneri*, *L. hilgardii*, *L. pentoaceticus*, *L. rudensis*, *L. brassicae-fermentatae*, *L. lycopersici*, *L. caucasicus*, *L. pastorianus*, *L. bifidus*, *L. acidophil-aerogenes*, *L. parvus*, *L. frigidus* and *L. malefermentans* were all represented. Neither the culture of *L. caucasicus* (CC1) nor the freshly isolated organism from kefir (K1) conformed to the description in *Bergey's Manual* of the type species of the genus *Lactobacillus*, but were similar to the *Betabacterium caucasicum* of Orla-Jensen (1919). Other cultures of recent isolation which were classified in group VII were from beer, Stilton cheese, silage, Jugoslavian sour milk, raw milk, sauerkraut and the bovine rumen. Four strains (SM1A, S38A, S40A, S41A) isolated from meat and sausages by Niven, Castellani & Allanson (1949) as well as seven other strains (T3, BR28, RM1, KR1, SL48, SL49, SL50) in this group were atypical in that they failed to produce ammonia from arginine.

Organisms of group VIII

Fifteen strains named *L. fermenti* (F1–F4, F6–F16), two named *L. gayonii* (G1, G2), one named *L. mannitopoeus* (M1) and thirteen freshly isolated organisms from Gruyère cheese, *présure*, pig faeces, calf stomach, chick duodenum, the human mouth and raw milk showed the reactions characteristic of group VIII. Only ten strains produced gas from citrate; heat tolerance was variable, some strains failing to survive 30 min. at 60° and others remaining viable after 90 min. at 65°.

Unclassified organisms

Section 1. Named strains of *L. lactis*, *L. bulgaricus*, *L. jugurt* and *L. helveticus* were among the twenty-four strains in this section. It is of interest that five organisms from the calf abomasum used to supply rennet for the manufacture of Gruyère cheese, and two strains (AH4, AH5) obtained from such cheese, belonged in this section. Four freshly isolated strains from silage and two from yoghourt were also included.

Section 2. The eleven cultures in this section (BF1, BF3, GF1, GF2, N3, N6, N7, O8, RF1, P28, P29) were of oral, faecal or intestinal origin and all save P28 and P29 had been isolated recently. Harrison & Hansen (1950*a*) obtained P28 and P29 from the caecal faeces of turkeys and identified them as *L. plantarum*. The characters of the organisms in this section resemble those of strains of *L. acidophilus* included in group I except that growth occurred in the presence of 4% sodium chloride. It is for this reason alone that the organisms were not included in group I.

Section 3. Strains A7, A11, C15, C27, C28, C30, D6, D7, O4 and P27 with two freshly isolated cultures, MR1 and MR3B, formed section 3. P27 was the motile strain of *L. plantarum* described by Harrison & Hansen (1950*b*).

Section 4. Section 4 comprised all cultures (15) which could not be included in the groups or sections described above. Only six (S3(1), S3(2), S4, S5, S6 and PF7) of the 452 lactobacilli in the collection showed bifid forms, and vigorous growth of these strains was obtained only when the cultures were incubated anaerobically. The testing of these six strains, five of which (S3(1), S3(2), S4, S5, S6) were received as *L. bifidus* and one (PF7), which was isolated from pig faeces, involved slight modification of the standard methods. Broth media were boiled for 30 min., cooled, inoculated and a 'Vasgarte' seal (Hirsch & Grinsted, 1953) poured on the surface of the broth. The use of anaerobic jars or similar apparatus was therefore unnecessary. The technique of determining gas production required no modification. Because of the morphological and anaerobic requirements of these six strains, it was considered advisable to include them in section 4 with the unclassified organisms, although some of the bifid organisms possessed reactions similar to those of organisms of group VIII.

DISCUSSION

The aim of the work described here was the broad division of the genus *Lactobacillus* into well-defined groups, since this was considered an essential preliminary to the intensive study and complete classification of these organisms. The investigation has resulted in the definition of eight such groups, six of which are homofermentative and two heterofermentative. Three homofermentative groups, I, II and III, though plainly separated from the remaining groups, are not so clearly distinct from each other. The results shown in Table 2 justify the separation of groups I, II and III, but confirmation, probably through the use of additional tests, is required. The similarities found between the reactions of strains of *L. acidophilus* and of *L. bulgaricus* contained in these three groups agree with most of the reports previously published.

The characteristics of some species in the groups described differ considerably from those reported by other workers; this is particularly true of growth temperatures. *L. bulgaricus* is generally recognized as a thermophilic organism and Orla-Jensen (1919) described one strain which grew at 52·5° although the usual maximum temperature was 50°. One of the differentiating reactions of strains in group II is the failure to grow at 45° and a representative strain of this group is a named culture, *L. bulgaricus* NCTC 2889 (B2).

It is of interest that two strains (J1 and J2) received as *L. jugurt*, and two freshly isolated strains from yoghourt (YOG1 and YOG4) all possess similar characters (see section 1, Table 2) but differ from the organisms included in groups II and III. *L. bulgaricus* is now considered to be the essential organism in yoghourt (Hiscox, 1951) but, if this be true, J1, J2, YOG1 and YOG4 should be typical strains of *L. bulgaricus* and the organisms in section 1 would therefore form a 'bulgaricus' group. Strains now in groups II and III would presumably form other groups, not 'bulgaricus', and most of the well-recognized strains of *L. bulgaricus*, some of which are of historical significance, would then be excluded from a 'bulgaricus' group. It is obvious that many more strains, isolated directly from typical samples of yoghourt, must be studied before this problem is solved.

It is possible that the unclassified thermophilic organisms (including J1, J2, YOG1 and YOG4) in section 1 (Table 2) belong to group V even though differences in heat survival have prevented their inclusion at this stage. The evolution of new tests for the subdivision of group V should prove profitable as there are indications that three species, *L. lactis*, *L. thermophilus* and *L. leichmannii*, are included therein. The precise taxonomic position and detailed characteristics of *L. leichmannii* are of great importance because of the widespread use of certain strains of this organism in microbiological assays.

Nine named strains of *L. delbrueckii* were tested, and seven of these were classified in five different groups; D1 in group III, D2 in group I, D3 and D5 in group VI, D4 and D8 in group IV, D9 in group V. D6 and D7 have been included in section 3 of the unclassified organisms. *Bergey's Manual* states that *L. delbrueckii* grows at 50°, but none of the nine strains tested (except D4, D8 and D9) grew even at 48°. Freshly isolated strains from cereal mash have not yet been examined.

The status of *L. bifidus* is also uncertain. Morphological examination of two strains from the National Collection of Type Cultures (S1 and S2) revealed no branching and these organisms grew well aerobically; they have been classified in group VII. Five further strains of *L. bifidus* (S3(1), S3(2), S4, S5 and S6) and one freshly isolated strain (PF7) showed bifid forms and grew vigorously only under anaerobic conditions; these have not been classified but are included in section 4.

The significance of the correlation between the production of gas from glucose and of ammonia from arginine is unknown, but it may be that organisms with both these properties possess a specialized metabolism. Examination of further cultures may indicate that organisms having one or other of these characteristics (e.g. LE3, S38A) belong to separate groups.

An examination of the morphological and colonial appearances of all strains, recorded under standard conditions, indicated that the differences between the characters of organisms within a given group and of organisms of separate groups were equally variable. At the outset of this work the sugar fermentation reactions of the ninety-five strains originally maintained in the N.I.R.D. collection were determined. Although thirty-one sugars were tested, it has not been possible to correlate any of the results with the groupings described in this paper. Morphology, colonial form and sugar fermentation reactions may be of value, however, when the groups have been further subdivided.

All replicate cultures have shown identical reactions in the physiological tests except A2 and A6 which have already been discussed. Duplication and repetition of the tests have given consistent results. The techniques are neither complicated nor in any sense laborious. All media are easily prepared and no elaborate apparatus is required, an accurate water-bath being the only necessary item of specialized equipment. The tests described satisfy therefore the requirements of simplicity, reproducibility and reliability.

The 197 freshly isolated cultures in the collection were obtained from a variety of sources including beer, silage, faeces, milk and cheese. One hundred and seventy (86·3 %) of these strains have been classified in the eight groups: a further twenty-one fall into the first three sections and only six organisms are included amongst the miscellaneous strains in section 4. It is of interest that the percentage (86·3) of freshly isolated cultures classified is identical with the proportion (86·3 %) of strains classified in the total collection. The grouping of so high a percentage of freshly isolated strains is of importance. It should now be possible to classify fresh isolations within existing groups, so that the undesirable practice of naming cultures according to their source rather than their characteristics may become less general.

The collection contains no representatives of the type species of the genus, *L. caucasicus* Beijerinck. Both the strain received as *L. caucasicus* (CC1) and the organism isolated from kefir (K1) were similar to *Betabacterium caucasicum* Orla-Jensen, and were classified in group VII. The ambiguity of these names appears to be contrary to Principle 1 of the International Code of Nomenclature (Buchanan, St John-Brooks & Breed, 1948) and the lack of representatives and detailed description of the type species suggests that the substitution of a recognizable and well-characterized lactobacillus as type species would be advisable. However, it is clear that further intensive study of the genus *Lactobacillus* is essential before a more suitable type species can be proposed.

Sherman (1937) described the characteristics used in the classification of streptococci and there is much in common between these biochemical tests and those employed in this work. Four tests in particular, namely growth at 10° and 40°, survival at 60°, production of NH_3 and growth in 6·5 % sodium chloride, are almost identical in each case.

The absolute classification of the homofermentative streptococci depends upon a combination of biochemical tests together with serological grouping. The classification of leuconostoc, which may be compared with the heterofermentative lactobacilli, has not been studied in detail. The reactions of the homofermentative lactobacilli resemble in many ways those of the homofermentative streptococci and it may be that a complete classification of the lactobacilli will eventually be based upon both biochemical and serological reactions. Some evidence in this direction has already been obtained. Dr M. Elisabeth Sharpe (personal communication), in preliminary work, has succeeded in preparing what appear to be grouping sera for certain species and a high degree of correlation has been found between these serological results and the grouping described in this paper.

The confusion existing within the genus *Lactobacillus* necessitated the grouping of strains before any given species could be selected for intensive study. This grouping has been accomplished and further investigation and subdivision of each group may now be undertaken. It is hoped that further work will lead to a complete classification of the genus.

I wish to thank Dr A. T. R. Mattick for his advice and guidance, and Mr L. F. Lawes for the preparation of the media. Dr T. Gibson kindly read the manuscript and made valuable suggestions. Thanks are due also to those who sent cultures either to Dr Mattick or to myself.

REFERENCES

Beijerinck, M. W. (1889). Sur le kéfir. *Arch. néerl. Sci.* **23**. 428.

Briggs, M. (1953). An improved medium for lactobacilli. *J. Dairy Res.* **20**, 36.

Buchanan, R. E., St John-Brooks, R. & Breed, R. S. (1948). International Bacteriological Code of Nomenclature. *J. Bact.* **55**, 287 reprinted in 1949, *J. gen. Microbiol.* **3**, 444.

Burri, R. & Kollmann, H. (1941). Studien über die Gattung Thermobacterium (Orla-Jensen) mit besonderer Berücksichtigung der Säuerungsflora des jungen Emmentalerkäses. *Landw. Jb. Schweiz*, **55**, 407.

Curran, H. C., Rogers, L. A. & Whittier, E. O. (1933). The distinguishing characteristics of *Lactobacillus acidophilus*. *J. Bact.* **25**, 595.

Davis, J. G. (1935). The use of special litmus milk media for the diagnostic culture of lactic acid bacteria. *J. Dairy Res.* **6**, 121.

Friedman, E. & Black, L. A. (1941). Some characteristics of '*Lactobacillus bifidus*' and its relation to *Lactobacillus acidophilus*. *J. Bact.* **42**, 152.

Gibson, T. & Abd-el-Malek, Y. (1945). The formation of carbon dioxide by lactic acid bacteria and *Bacillus licheniformis* and a cultural method of detecting the process. *J. Dairy Res.* **14**, 35.

Grigoroff, S. (1905). Étude sur un lait fermenté comestible. Le 'Kisselo-mleko' de Bulgarie. *Rev. méd. Suisse rom.* **25**, 714.

Hadley, F. P., Bunting, R. W. & Delves, E. A. (1930). Recognition of *Bacillus acidophilus* associated with dental caries: a preliminary report. *J. Amer. dent. Ass.* **17**, 2041.

Harrison, A. P. & Hansen, P. A. (1950*a*). Lactobacilli from turkeys. *J. Bact.* **60**, 543.

Harrison, A. P. & Hansen, P. A. (1950*b*). A motile lactobacillus from the cecal feces of turkeys. *J. Bact.* **59**, 444.

Heinemann, P. G. & Hefferan, M. (1909). A study of *B. bulgaricus*. *J. infect. Dis.* **6**, 304.

Hirsch, A. & Grinsted, E. (1953). Media for the growth and enumeration of anaerobic spore formers from cheese with observations on the effect of nisin. *J. Dairy Res.* In the Press.

Hiscox, E. R. (1951). A note on yoghourt. *Proc. Soc. appl. Bact.* **14**, xii.

Howe, P. R. & Hatch, R. E. (1917). A study of the microorganisms of dental caries. *Dent. Cosmos*, **59**, 961.

Kulp, W. L. (1929). Preliminary report on a method for the differentiation of *L. acidophilus* from *L. bulgaricus*. *J. Bact.* **17**, 355.

Kulp, W. L. & Rettger, L. F. (1924). Comparative study of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. *J. Bact.* **9**, 357.

McIntosh, J., James, W. W. & Lazarus-Barlow, P. (1922). An investigation into the aetiology of dental caries. I. The nature of the destructive agent and the production of artificial caries. *Brit. J. exp. Path.* **3**, 138.

McIntosh, J., James, W. W. & Lazarus-Barlow, P. (1924). An investigation into the aetiology of dental caries. II. The biological characteristics and distribution of *B. acidophilus odontolyticus.* III. Further experiments on the production of artificial caries. *Brit. J. exp. Path.* **5**, 175.

Morishito, T. (1929). Studies on dental caries, with special reference to aciduric organisms associated with this process. I. Isolation and description of organisms. *J. Bact.* **18**, 181.

Moro, E. (1900). Über den *Bacillus acidophilus. Jb. Kinderheilk.* **52**, 38.

Niven, Jun., C. F., Smiley, K. L. & Sherman, J. M. (1942). The hydrolysis of arginine by streptococci. *J. Bact.* **43**, 651.

Niven, Jun., C. F., Castellani, A. G. & Allanson, V. (1949). A study of the lactic acid bacteria that cause surface discolorations of sausages. *J. Bact.* **58**, 633.

Orla-Jensen, S. (1919). *The lactic acid bacteria.* Copenhagen: Andr. Fred. Host and Son.

Orla-Jensen, S. (1942). *The lactic acid bacteria.* 2nd. ed. Copenhagen: Ejnar Munksgaard.

Orla-Jensen, S. (1943). *The lactic acid bacteria.* Ergänzungsband. Copenhagen: Ejnar Munksgaard.

Orla-Jensen, S., Orla-Jensen, A. D. & Winther, O. (1935–36). *Bacterium bifidum* und *Thermobacterium intestinale. Zbl. Bakt.* 2 Abt. **93**, 321.

Pederson, C. S. (1938). The gas-producing species of the genus *Lactobacillus. J. Bact.* **35**, 95.

Pederson, C. S. (1948) in *Bergey's Manual of Determinative Bacteriology* (6th ed.), p. 349. Ed. by Breed, R. S., Murray, E. G. D. & Hitchens, A. P. London: Baillière, Tindall and Cox.

Rahe, A. H. (1918). The classification of the aciduric bacteria. *J. Bact.* **3**, 407.

Rogosa, M. (1946). Significance in nutritional research of correct identification of *Lactobacillus casei, L. delbrueckii* and *L. bulgaricus. J. Bact.* **51**, 575.

Rosebury, T., Linton, R. W. & Buchbinder, L. (1929). A comparative study of dental aciduric organisms and *Lactobacillus acidophilus. J. Bact.* **18**, 395.

Sherman, J. M. (1937). The streptococci. *Bact. Rev.* **1**, 3.

Sherman, J. M. & Hodge, H. M. (1940). The value of certain tests in the differentiation of *Lactobacillus bulgaricus* from *Lactobacillus acidophilus. J. Bact.* **40**, 11.

Sherwood, I. R. (1939). The bacterial flora of New Zealand Cheddar cheese. *J. Dairy Res.* **10**, 426.

Shimwell, J. L. (1948). A rational nomenclature for the Brewery lactic acid bacteria. *J. Inst. Brew.* **54**, 100.

Tissier, H. (1900). *Recherches sur la flore du intestinale normale et pathologique du nourisson.* Thèse. Université de Paris.

Tittsler, R. P., Geib, D. S. & Rogosa, M. (1947). Taxonomy of the genus *Lactobacillus* with special reference to correlations of differential characteristics. *J. Bact.* **54**, 12.

Tittsler, R. P., Pederson, C. S., Snell, E. E., Hendlin, D. & Niven, Jun., C. F. (1952). Symposium on the lactic acid bacteria. *Bact. Rev.* **16**, 227.

Weaver, R. H. (1932). Fermentation studies on *Lactobacillus acidophilus* and *Lactobacillus bulgaricus. J. infect. Dis.* **51**, 273.

Weiss, J. E. & Rettger, L. F. (1934). *Lactobacillus bifidus. J. Bact.* **28**, 501.

(*Received* 19 *March* 1953)

PICKETT, M. J. & CABELLI, V. J. (1953). *J. gen. Microbiol.* 9, 249–256.

The Precipitating Antigens of Friedländer's Bacillus

BY M. J. PICKETT AND V. J. CABELLI*

From the Department of Bacteriology, University of California,
Los Angeles, California

SUMMARY: The biochemical characteristics of approximately eighty strains of Friedländer's bacillus are described. Certain aspects of their antigenic patterns were studied by testing the precipitating antigens of phase variants of originally mucoid strains, as well as the parent strains themselves. Evidence is presented that an as yet undescribed antigenic layer exists between those characteristic of the mucoid and smooth phases. This layer is most pronounced in the Sm phase organisms. The Sm antigen was shown to be present in nearly all Friedländer's strains and absent in the *Bacterium coli* strains studied; hence an Sm antiserum should be valuable for diagnostic purposes. The biochemical and serological results are discussed in regard to the taxonomy of this group with particular reference to the consolidation, already proposed by other workers, of the genera *Klebsiella* and *Aerobacter*.

In the studies to be described the precipitin patterns of phase variants of originally mucoid organisms were used to demonstrate another antigenic layer in addition to those described by Kauffmann (1949*a*) and to show the possibility of its use in the identification of members of the *Klebsiella* group. Edwards (1929), Julianelle (1930), Osterman & Rettger (1941*b*), and Kauffmann (1949*a*) studied the biochemical characteristics of the group (for earlier work see *Topley and Wilson's Principles of Bacteriology*, 1946). Their findings indicate an extreme variability in the biochemical reactions. Kauffmann (1949*a*) listed some general biochemical characteristics of the group. He defined the *Klebsiella* group as 'non-motile, Gram-negative, non-sporing rods that do not form indole, and which ferment adonitol, inositol and other carbohydrates or alcohols, often breaking down urea and frequently giving a positive Voges-Proskauer (VP) reaction and negative methyl-red (MR) reaction'. He went on to mention that the variable characteristics of the entire family Enterobacteriaceae make it necessary that all the above characteristics, rather than any one of them, be used in classifying these organisms.

Julianelle (1926*a*, 1926*b*) first demonstrated the antigenic specificity of the capsular material and classified the organisms on that basis. Kauffmann (1949*a*) reported fifteen different serological groups on the basis of the specificity of the capsular K antigen, and three main types on the basis of an antigenic carbohydrate underlayer (O antigen). The close serological relationship of *Klebsiella pneumoniae* to *Aerobacter aerogenes* has been demonstrated by Osterman & Rettger (1941*a*, *b*) and by Kauffmann (1949*a*). Shared O antigens have been demonstrated between *K. pneumoniae* and *Bact. coli* by

---

* Present address: Department of Bacteriology and Preventive Medicine, School of Medicine, University of Missouri, Columbia, Missouri.

Kauffmann (1949*a*). Henriksen (1949), who used the complement fixation test, found cross reactions between mucoid strains of *K. pneumoniae* and *Bact. coli*; however, no such reactions were obtained when he used the agglutination test.

MATERIALS AND METHODS

The organisms which were used in these studies consisted of stock strains of *K. pneumoniae* from our collection and of strains recently isolated from sputa and urines. Some of the stocks were copies of those used by Julianelle and by Kauffman. The strains were as follows:

Isolated from blood or sputum

Type A:
- SA1 Julianelle's Sc strain, American Type Culture Collection no. 4208.
- SA2 Levy-Bruhl's Bonneral strain.
- SA3 Julianelle's Murtens strain.
- SA4, SA5, SA6, SA7.

Type B:
- SB1 From Dr Gordon Nichols.
- SB2 From New England Deaconess Hospital.
- SB3 Julianelle's 'E' strain, pneumonia in a guinea pig.
- SB4, SB5.

Type C:
- SC1 Julianelle's F-10 strain.
- SC2, SC3, SC4.
- K11 Kauffmann's 5056 strain.
- SX1 Group 'X'.

Isolated from urine

- UA1 Type A.
- UB1, UB2, UB3 Type B.
- UC1 Type C.
- UX1–36 Group 'X'.
- K1 Kauffmann's 1193 strain.
- K2 Kauffmann's K390 strain.
- K3 Kauffmann's K56 strain.
- K4 Kauffmann's 1015 strain.
- K6 Kauffmann's 373 strain.
- K8 Kauffmann's 919 strain.
- UI1–10 Indole positive.

Rhinoscleroma and Ozaenae strains

- K5 Kauffmann's Rhinoscleroma strain 5046—type C.
- K9 Kauffmann's Ozaenae strain 5050—type D.
- K10 Kauffmann's Ozaenae strain 5052—type F.
- K12 Kauffmann's Ozaenae strain 5051—type E.

Cells from a single colony on a peptone agar plate were subcultured to peptone broth and then to the various test media. The MR and VP tests were read after 36 hr. The rest of the tests were read after 24 hr. and at 1 day intervals thereafter to the 15th day.

The strains were characterized serologically by the use of the interphase precipitin test. Growth from an 18 hr. peptone agar slope culture was emulsified in approximately 10 ml. saline and centrifuged at 3000 r.p.m. until a clear supernatant was obtained. The supernatant containing the extracted material was layered by means of a capillary pipette over undiluted antisera in tubes whose inside diameters were about 3 mm. The tubes were left at room temperature and read at 5 and 30 min. intervals. This procedure was used for the mucoid parent strains as well as for the phase variants. In order to extract the Sm antigen from mucoid organisms (Table 3, labelled (heat)), the cell suspensions were heated at 100° for 7 min. prior to centrifugation. A positive test manifested itself as a precipitate at the interface. Positive and saline controls were performed with each run.

All the antisera used in this study were prepared by injecting rabbits with increasingly heavy 0·4 % phenolized saline suspensions of unwashed cells grown on peptone agar slopes. The antigens were injected every other day for 3 months. Strains SA1, SB2 and SC1 were used to prepare anti-A, anti-B, and anti-C sera, respectively. Anti-Sm was a bivalent serum against strains UX35 and UX36. In the preparation of anti-Sm serum, however, washed cell suspensions were used. Unwashed and unheated cell suspensions of SB2s, a smooth variant of SB2 which had lost its ability to react with anti-B or anti-Sm sera but which had retained the O antigenic layer, were used for the preparation of the anti-O serum.

The colonial and microscopic morphologies of the stock strains and the variants which were obtained in the course of repeated passage through brilliant green were studied, and their antigenic precipitin patterns were determined by using the sera mentioned above. In order to obtain the variants, the types A, B, and C parent strains were inoculated in duplicate to 16 mm. tubes containing 10 ml. of a medium composed of 1 % peptone, 0·5 % NaCl, and brilliant green to a concentration of $1:10^7$ in distilled water; the tubes were then incubated at 37°. Subcultures were made every other day to the medium mentioned above; however, the concentration of the dye was increased when good growth was obtained in the previous subculture. A 0·1 ml. inoculum was generally used; but, when no growth was obtained in a higher concentration of the dye, the amount was increased up to 1·0 ml. At weekly intervals samples were taken and plated on peptone agar for the detection of the variants which were then picked off and replated to the same medium. They were so replated on at least four successive days at the end of which time they were streaked on peptone agar slopes and kept in stock for later use. At the end of 3 months, when a representative number of variants had been obtained and when all the parent strains were growing in a 1:50,000 concentration of brilliant green, the collected variants were replated on peptone agar from which representative colonies were restreaked to slopes for the precipitin tests.

RESULTS

The variability in most of the biochemical reactions can be seen from the results summarized in Table 1. Maltose, sucrose, galactose, mannitol, sorbitol, mannose, xylose and arabinose were almost always fermented in 24 hr. Most of the strains which were isolated from urine resembled UX1 and UX7 both biochemically and serologically.

We would like to point out that the lactose-negative strains are much less reactive biochemically than the lactose-positive ones. The lactose-negative strains have almost or completely lost the ability to produce acetylmethylcarbinol and to ferment dulcitol and inositol; it was also found that they were unable or only weakly able to use ammonia as a source of nitrogen.

Table 1. *Biochemical characteristics of the strains of Friedländer's bacillus examined*

Strain	MR	VP	Ind	Inos	Dulc	Lact
SA1, 3	+	+	−	+10d	−	−
SA2, 5	−	+	−	+	+	+
SA4, 6	+	−	−	+10d	−	−
SA7	−	+	−	+	−	+
SB1	±	±	−	+	−	+
SB2	±	±	−	+2d	−	+
SB3, 4	−	+	−	+	−	+
SB5	−	+	−	+	−	+
SC1, 2	−	+	−	+	+	+
SC3	+	−	−	+	+	+
SC4	−	+	−	+6d	−	+2d
SX1	−	+	−	+	+	+
UA1	−	+	−	+	+	+
UB1, 2	−	+	−	+	−	+
UB3	−	+	−	+	−	+
UC1	±	±	−	+	+	+
UX2	+	+	−	−	−	+3d
UX3	+	±	−	+	+	+
UX4	−	+	−	+	+	+
UX7	+	−	−	+	−	+
UX14	+	+	−	+	+	+
UX35	−	+	−	+	−	+
UX36	+	−	−	+2d	−	+
K1, 2, 3	−	+	−	+	−	+
K4, 6	−	+	−	+	−	+
K5	+	−	−	+	−	−
K8	−	+	−	+	+	+
K9	+	−	−	+	−	−
K10	+	−	−	+2d	−	−
K11	+	−	−	+2d	+	+
K12	−	+	−	+	+	+

MR = Methyl red; VP = Voges-Proskauer; Ind = Indole; Inos = Inositol; Dulc = Dulcitol; Lact = Lactose; d = days; + = posititive; − = negative; ± = slightly positive.

The serological characteristics of the strains are presented in Table 2. We would like to call special attention to the almost uniform reaction with the bivalent serum, anti-Sm. All the strains of *Bact. coli* which were tested were uniformly negative with the anti-Sm serum.

The colonial morphologies of the phase variants are to be seen in Pl. 1, figs. 1–5. Microscopically, coccobacilli were the predominant forms in the mucoid phase. Rough phase variants were composed of numerous long rods, swollen forms, and long filaments, some of which extended the length of the visual field. Phase variants between these two extremes showed gradations in form consistent with the colonial morphology by which they were designated.

The precipitin pattern of the phase variants of strains SA1, SB2, SC2, UX35 and UX36 are shown in Table 3. The latter two strains are those used to prepare the bivalent serum. The results point to the existence of at least three distinct soluble antigenic layers, in addition to those possessed by the rough phase organisms.

Table 2. *Serological characteristics of the strains of Friedländer's bacillus examined*

	Antisera			
Strain	A	B	C	Sm
SA1, 3	+	–	–	+
SA2, 5	+	–	–	+
SA4, 6	+	–	–	+
SA7	+	–	–	+
SB1	–	+	–	+
SB2	–	+	–	+
SB3, 4	–	+	–	+
SB5	–	+	–	+
SC1, 2	–	–	+	+
SC3	–	–	+	+
SC4	–	–	+	+
SX1	–	–	–	+
UA1	+	–	–	+
UB1, 2	–	+	–	+
UB3	–	+	–	–
UC1	–	–	+	+
UX2	–	–	–	+
UX3	–	–	–	+
UX4	–	–	–	+
UX7	–	–	–	+
UX14	–	–	–	+
UX35	–	–	–	+
UX36	–	–	–	+
K1, 2, 3	–	–	–	+
K4, 6	–	–	–	+
K5	–	–	–	+
K8	–	–	–	+
K9	–	–	–	+
K10	–	–	–	+
K11	–	–	+	+
K12	–	–	–	+

– = no precipitate using the raw antiserum.
+ = the formation of a precipitate at the interphase.

DISCUSSION

The results leave no doubt that biochemical tests do not offer a very satisfactory basis for the differentiation of the genus *Klebsiella* from the genus *Bacterium* (*Escherichia*). Of them all, the indole reaction and the fermentation of inositol as exclusion tests seem the most reliable. The authors feel, as did Kauffmann (1949*a*, 1949*b*) that the genera *Klebsiella* and *Aerobacter* should be fused into a single genus since they can be differentiated neither biochemically nor serologically.

The serological identification on the basis of capsular antigens has been quite adequately covered by Kauffmann (1949*a*); the large number of types

Table 3. *Precipitin reactions of phase variants*

		Antisera				
Strain	Phase	A	B	C	Sm	O
SA1	m	++++	·	·	–	–
SA1	ms	+	·	·	+++	+++
SA1	sm	+	·	·	++	++++
SA1	s	±	·	·	–	+++
SA1	r	–	·	·	–	–
SB2	m	·	++++	·	+	–
SB2	m (heat)	·	++++	·	++++	–
SB2	sm	·	++	·	+++	+++
SB2	s	·	–	·	–	++
SB2	r	·	–	·	–	–
SB3	m	·	++++	·	–	–
SB3	m (heat)	·	++++	·	+++	–
SB3	sm	·	++	·	++++	+++
SB3	s	·	–	·	–	+++
SB3	sr	·	–	·	–	++
SC2	m	·	·	++++	–	–
SC2	ms	·	·	+++	+++	++
SC2	s	·	·	–	–	+++
SC2	rs	·	·	–	–	+
UX35	m	–	–	–	+++	–
UX35	m (heat)	·	·	·	+++	++
UX36	m	·	·	·	+++	–
UX36	m (heat)	·	·	·	+++	–
UX36	ms	·	·	·	+++	–
UX36	s	·	·	·	–	++
UX36	sr	·	·	·	–	++
UX36	r	·	·	·	–	–

Heat = suspension heated at 100° for 7 min. prior to centrifugation.

++++ to – = strong to negative precipitate at the interphase.

m = mucoid; ms = mucoid-smooth; sm = smooth-mucoid; s = smooth; sr = smooth-rough; rs = rough-smooth; r = rough.

O = agglutination with appropriate O sera.

and the discovery of new ones (Miss Kelly, personal communication) makes identification by this means impractical. It was to this end that the bivalent serum was used in the hope that it could be utilized as an easy means of identification and classification. To be of value the serum would have to react with all mucoid and smooth-mucoid strains of *K. pneumoniae* and give negative precipitin tests with all other species. It is felt that the first criterion has been satisfied, but more extensive tests with related organisms, i.e. *Bacterium* (*Escherichia*) spp., *Proteus* spp., etc., are necessary before a final statement on the second point can be made.

Although absorption tests would be needed definitely to prove the identity of the precipitating antigens of *K. pneumoniae*, certain conclusions can be drawn as to their nature as seen from the data of Table 3:

1. The Sm antigen or antigens are distinct from and lie beneath the capsular A, B, and C antigens. This is shown by the fact that anti-Sm serum will not precipitate the supernatant from mucoid A, B or C strains, but will precipitate the supernatant from all three if the cell suspensions are first heated.

2. The Sm antigen or antigens are distinct from the O antigens. This is borne out by the fact that Sm antiserum but not O will react with the supernatant from heated suspensions of mucoid phase organisms, while the O and not the Sm antiserum will react with that from smooth phase cells.

3. In view of the preceding it can be said that there are at least three distinct antigenic layers exclusive of the one characteristic of the rough phase.

In view of the peculiarities of the Sm antigen, one can see why it could have been overlooked by previous workers. Since it is absent in the smooth organisms, anti-smooth sera would not contain antibodies against it. Antibodies against it may be present in anti-mucoid sera, but the suspensions would have to be heated before testing the supernatant in order to obtain a positive precipitin reaction. Conceivably, antibodies against the Sm antigen could be absent from anti-(m)sera if the cell suspension is neither heated nor washed before injection, if it is used as soon as prepared, or if the immunization time is short.

Finally, the ability to detect the Sm antigen was dependent upon the fortuitous finding of strains UX35 and UX36 in which the Sm antigen was readily exposed by washing alone (Table 3). Hence, anti-Sm antibodies were produced when only washed cell suspensions were used as the immunizing antigen.

REFERENCES

Edwards, P. R. (1929). Relationships of the encapsulated bacilli with special reference to *Bact. aerogenes*. *J. Bact.* **17**, 339.

Henriksen, S. D. (1949). Sero-types of a collection of mucoid Gram-negative rods. *Acta path. microbiol. scand.* **26**, 424.

Julianelle, L. A. (1926*a*). A biological classification of *Encapsulatus pneumoniae* (Friedländer's bacillus). *J. exp. Med.* **44**, 113.

Julianelle, L. A. (1926*b*). Immunological relationships of encapsulated and capsule-free strains of *Encapsulatus pneumoniae* (Friedländer's bacillus). *J. exp. Med.* **44**, 683.

Julianelle, L. A. (1930). The distribution of Friedländer's bacilli of different types. *J. exp. Med.* **52**, 539.

Kauffmann, F. (1949*a*). On the serology of the *Klebsiella* group. *Acta path. microbiol. scand.* **26**, 381.

Kauffmann, F. (1949*b*). On the classification of the Enterobacteriaceae. *Acta path. microbiol. scand.* **26**, 879.

Osterman, E. & Rettger, L. F. (1941*a*). A comparative study of organisms of the Friedländer and coli-aerogenes groups. I. Morphological and cultural characteristics, with emphasis on variation. *J. Bact.* **42**, 699.

Osterman, E. & Rettger, L. F. (1941*b*). A comparative study of organisms of the Friedländer and coli-aerogenes groups. II. Pathogenicity, biochemical reactions, and serological relationships. *J. Bact.* **42**, 721.

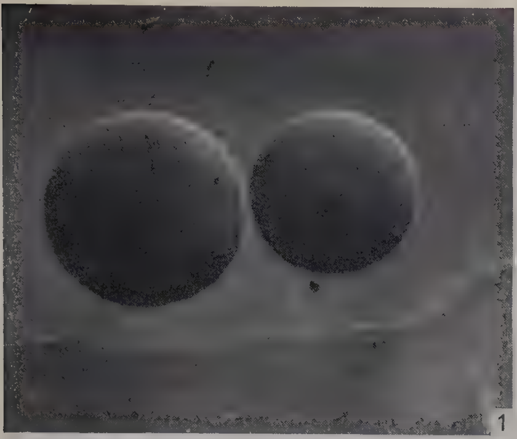
Topley and Wilson's Principles of Bacteriology and Immunology (1946). 3rd ed. Revised by Wilson, G. S. & Miles, A. A. Baltimore: Williams & Wilkins Co.

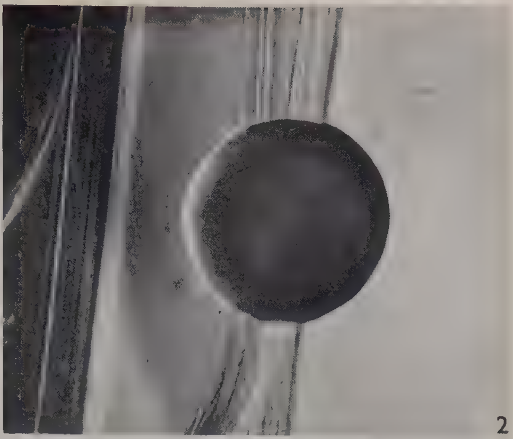
EXPLANATION OF PLATE

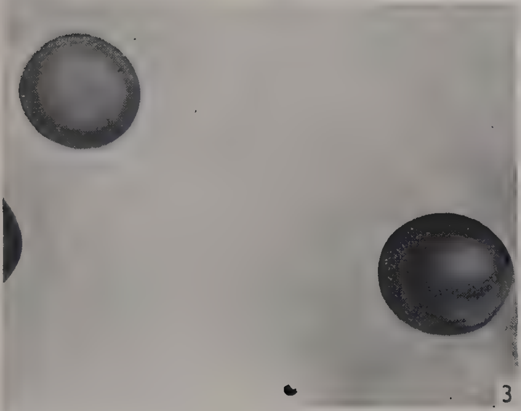
Colonial characteristics of phase variants of *Klebsiella pneumoniae* strain SA1 on peptone agar. × 7.

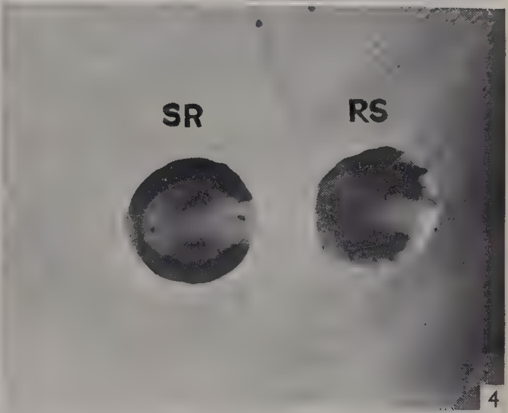
Fig. 1. Mucoid colonies.
Fig. 2. Smooth-mucoid colony.
Fig. 3. Smooth colonies.
Fig. 4. Smooth-rough and rough-smooth colonies.
Fig. 5. Rough colonies.

(*Received* 23 *March* 1953)

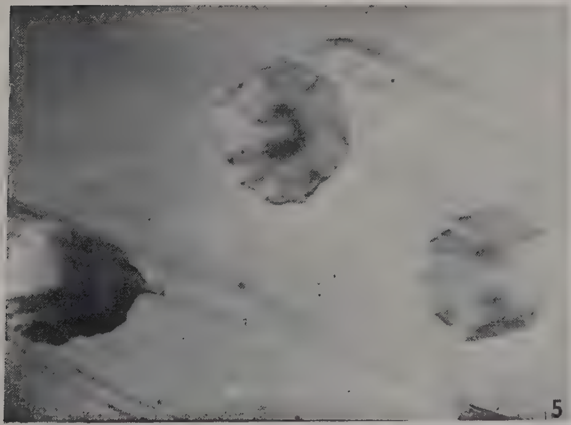

1


2


3



4


5

M. J. Pickett & V. J. Cabelli—Antigens of Friedländer's bacillus. Plate 1

MITCHELL, P. & MOYLE, J. M. (1953). *J. gen. Microbiol.* 9, 257–272.

Paths of Phosphate Transfer in *Micrococcus pyogenes*: Phosphate Turnover in Nucleic Acids and other Fractions

BY P. MITCHELL AND JENNIFER M. MOYLE

Department of Biochemistry, University of Cambridge

SUMMARY: The paths of transfer of phosphate groups during uptake of inorganic orthophosphate by *Micrococcus pyogenes* var. *aureus* (strain Duncan) have been studied by following the incorporation of ^{32}P-labelled phosphate groups into the phospholipid (LP), acid-soluble inorganic (AI) and organic (AO), deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and glycerophospho-protein complex (PGP) fractions of the cells.

In resting cells a strictly reciprocal exchange of inorganic phosphate across the osmotic barrier is half complete in 70 min. During respiration or growth, phosphate moves inward through the osmotic barrier at the same rate as during rest, but the outward movement is abolished. Phosphate accumulates mainly in the AI, AO and LP during respiration, but in all the fractions during growth. Most of the phosphate of the organic fractions is drawn through the AI. The RNA is formed from the nucleotides of the AO, while the DNA phosphate is derived either directly from the AI or from intermediates in the AO. Little or no turnover of RNA or DNA phosphate occurs during growth, and phosphate is not transferred directly or indirectly from RNA to DNA or vice versa. The PGP, although the major constituent of the mechanically rigid cell envelope, does not participate as a whole in phosphate transfer reactions during rest or respiration; but during growth the amount of PGP increases in proportion to the expansion of cell envelope area. The LP phosphate exhibits rapid turnover during respiration and growth.

The work to be described here was undertaken to obtain information about the process of assimilation of inorganic orthophosphate by *Micrococcus pyogenes* var. *aureus* (strain Duncan). The aim was to trace with ^{32}P the paths of entry of phosphate from the suspension medium into the main phosphate fractions of the cells when (*a*) resting, (*b*) respiring, and (*c*) growing. The phosphate fractions selected for study were: the cold 5 % trichloroacetic acid-soluble inorganic (AI) and organic (AO), the phospholipid (LP), deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and glycerophospho-protein complex (PGP) fractions of the cells; and the inorganic phosphate of the medium (mI). All are well known except the PGP, the major constituent of the mechanically rigid cell envelope of *M. pyogenes*, formerly called XSP (Mitchell & Moyle, 1951*b*). Essentially the problem was the same as that of Juni, Kamen, Reiner & Spiegelman (1948) in their studies of phosphate assimilation by baker's yeast. Our treatment differs, however, in one important respect. Spiegelman and his collaborators interpreted their findings in the usual terms of phosphate 'turnover' inferred in many cases from determinations of relative specific activity alone. Much more definite information may be obtained from estimations of the total P and ^{32}P contents of the phosphate fractions: moreover, interpretation becomes easier and less ambiguous because the total P and ^{32}P contents each possess the property, not shared by the values of relative specific activity, of being additive. Our experiments were therefore designed to make the best use of the following three principles:

(i) the P content of unit volume of the system (cells + suspension medium) remains constant;

(ii) the ^{32}P content of unit volume of the system remains constant;

(iii) the ratio of ^{32}P to P passing out of a given phosphate fraction is equal to the ratio of ^{32}P to P in that fraction.

The validity of these principles is initially dependent on an arrangement of the experimental conditions during phosphate assimilation such that any changes of P content or volume of the cell suspension as a whole are known. To utilize the principles it must be possible to determine the total P and ^{32}P contents of the phosphate fractions of the cells at intervals during the progress of assimilation. A fractionation procedure must, therefore, be used in which the phosphate fractions can be separated quantitatively or in known yield in a known state of purity. Principle (ii) also depends upon the duration of the experiment being short in comparison to the half-life of the ^{32}P (14·3 days) or upon the application of corrections for decay. Principle (iii) requires special consideration. This principle depends upon the assumption that the phosphate passing out of a fraction by a given metabolic path may be regarded as a random sample of the phosphate of the whole fraction. The validity of this assumption will be considered when principle (iii) is applied.

METHODS

Growth and preparation of organism. Cultures of *M. pyogenes* were grown at 25° in 2 l. of casein digest medium containing 1 % (w/v) glucose and 0·1 % Marmite in 5 l. rotated flasks with air exchange heads (Mitchell, 1949). The cells were harvested at a concentration equivalent to 2 mg. dry-wt./ml., washed twice with distilled water and once with the salt solution used in the assimilation experiments.

Assimilation of phosphate. The cells, prepared as described above, were suspended at a concentration equivalent to 10 mg. dry-wt./ml. in a salt solution containing 5 μmole Na_2HPO_4, 5 μmole NaH_2PO_4, 100 μmole NaCl, 10 μmole KCl and 3·3 μmole $MgCl_2$/ml. Two hundred ml. of the suspension was transferred to a 1 l. rotated flask with an air exchange head and allowed to equilibrate at 25° for 20 min. The inorganic phosphate of the medium was now labelled by adding sodium phosphate containing ^{32}P of total activity *c.* 200 μC. in 1 ml. of the salt solution. A reference sample of 1 ml. of the suspension was immediately withdrawn for determinations of the total P and ^{32}P contents per ml. of the system. At the same time as the reference sample and subsequently at suitable intervals 20 ml. samples were taken for determinations of the P and ^{32}P contents of the suspension medium and of the fractions of the cells.

Ninety minutes after adding the ^{32}P to the resting cell suspension, respiration or growth was initiated in different experiments by adding 10 ml. of the salt solution containing glucose to give a final concentration of 5 % (w/v) or

10 ml. of the salt solution containing glucose and casein hydrolysate (vitamin-free casein hydrolysate of Ashe Laboratories) to give final concentrations of 5 % and 1 % (w/v) respectively. The 20 ml. samples of the suspension withdrawn during the progress of assimilation were immediately centrifuged. The suspension medium was centrifuged again after decanting from the pad of cells, and 1 ml. of the clear medium was diluted for estimations of P and ^{32}P, for determinations of glucose by the method of Nelson (1944) and for measurement of pH with a glass electrode. The pH value remained within the range 6·7–7·0, and the concentration of glucose did not fall below 4·8 %. The cells were washed three times with 20 ml. lots of distilled water, cooled in liquid air and dried over P_2O_5 in high vacuum at room temperature. Estimations of the turbidity of the washings showed that less than 1 % of the cells were lost, and thus the weights of the dry cell samples could be used to measure the amount of growth.

Separation of phosphate fractions

The procedure adopted for separating the phosphate fractions of whole cells and cell components was the same as that previously described (Mitchell & Moyle, 1951*b*) except that the trichloroacetic acid-soluble fraction was separated into organic and inorganic components. To 5 ml. of the acid-soluble fraction, 0·1 ml. of 20 N-ammonia and 0·1 ml. of 5 % (w/v) $MgCl_2, 5H_2O$ were added, and the magnesium ammonium phosphate was allowed to precipitate at 2° during 15 hr. The precipitate was centrifuged and washed three times with 1 ml. lots of N-ammonia, the supernatants, containing the acid-soluble organic phosphate, being collected and made up to 10 ml. with distilled water. The precipitate, containing the inorganic phosphate, was dissolved in 0·5 ml. of N-HCl and made up to 10 ml. with distilled water. Estimations of P showed that mutual contamination of AI and AO did not exceed 3 %.

Labile phosphate compounds occur as major components of the RNA and DNA fractions of baker's yeast (Juni *et al.*, 1948). Using the methods of Spiegelman and his collaborators, we were unable to separate significant amounts of labile phosphate compounds from the RNA or DNA fractions of *M. pyogenes*. Moreover, the amounts of 'ten minute' phosphate in these fractions were too small to be distinguished from the more labile phosphate of the nucleic acids, and the amounts of DNA measured by the extinction at 260 mμ., using a mean mononucleotide molecular extinction coefficient of 10,000, agreed with the amounts measured by the phosphate content to within ± 3 %. We concluded that quantities of labile phosphate compounds not exceeding 3 % of the total phosphate of the RNA and DNA fractions might be present. Less than 0·1 % of the phosphate of the RNA fraction occurred as inorganic phosphate, and hence the amounts of 'phosphoprotein' (Schmidt & Thannhauser, 1945) were considered to be negligible.

Glycerophosphoprotein complex fraction. When the above fractionation procedure was carried out on whole cells, it was not possible to separate the phosphate of the RNA and PGP fractions, and consequently separate estimations of the ^{32}P contents of these fractions could not be made. It was possible, however, to take advantage of the fact that some 80 to 90 % of the PGP can be obtained practically uncontaminated by RNA in the particulate fractions of mechanically disintegrated cells. The cells were separated into soluble and particulate (envelope + small particle) components by a method similar to that previously described (Mitchell & Moyle, 1951*b*). The distribution of P and ^{32}P in the cell components thus obtained from 50 mg. of dry cells was determined by the procedure applied to whole cells.

The (RNA + PGP) fraction of the particulate cell component contained less than 3 % of RNA phosphate. Measurements of the P and ^{32}P contents of this fraction were therefore taken to represent PGP phosphate within experimental error. On the other hand, the (RNA + PGP) fraction of the soluble cell component contained about 20 % of PGP phosphate, at least three-quarters of which consisted of particulate material carried over from the particulate fraction during decantation of the centrifugates. By taking precautions which were unnecessary in the routine of the present studies, the amount of PGP present in the (RNA + PGP) fraction of the soluble cell component could be diminished to less than 5 %. Assuming the ratio of ^{32}P to P estimated in the PGP of the particulate cell component represented that of the whole PGP, the ^{32}P content of the PGP of the (RNA + PGP) fraction of the soluble cell component could be estimated, and hence the total ^{32}P contents of the RNA and PGP of the cells computed.

It was necessary to consider that significant changes might occur in the P and ^{32}P contents of the RNA and PGP during disintegration of the cells. The ^{32}P contents of these fractions would, for instance, be seriously affected if exchange of phosphate occurred with the AI or AO. In order to assess the extent of such changes, the P and ^{32}P contents of all the phosphate fractions were compared throughout an experiment corresponding to that of Table 1. Systematic differences exceeding 5 % did not occur either in the P or ^{32}P contents of any of the fractions. In particular, the P and ^{32}P contents of the (RNA + PGP) fraction of the whole cells showed a standard deviation of only ± 3 % from the sum of the (RNA + PGP) contents of the soluble and particulate components. Thus, all significant changes of composition during disintegration were ruled out with the possible exception of exchange of phosphate between the RNA and PGP. The fact that the ratio of ^{32}P to P found in the RNA and PGP varied independently during the course of phosphate assimilation (Fig. 2) showed, however, that an exchange of phosphate between these fractions might occur during disintegration only to a small extent if at all.

Estimation of total P *and* ^{32}P. For determination of the total organic and inorganic phosphate contents we used micro-adaptations of the methods of Fiske & Subbarow (1925), giving values with a standard deviation of ± 1 % for samples containing 3 to 30 μg. of phosphorus.

The relative ^{32}P contents of the fractions were estimated as counts/min./μg. P in samples dried on nickel planchettes using a conventional end-window Geiger-Mueller counter and scaling unit, following the methods of Boursnell, Coombs & Rizk (1953). Triplicate or duplicate samples were taken for each determination and at least 1000 counts were registered for each sample. With every group of ten samples, the count given by the reference sample was redetermined so that corrections could be made for periodic changes in the efficiency of the counting machinery and for decay of the ^{32}P. The standard deviation of the ^{32}P measurements was *c.* ±5 %.

Indices of phosphate contents. The specific activities as counts/min./μg. P were converted to relative specific activities (or relative specific ^{32}P contents), R, defined as $R=k.{}^{32}\mathrm{P}/\mathrm{P}$, the constant k being fixed to give $R=1$ for the ratio of ^{32}P to P in the whole system. It is usual to define the unit of relative specific activity with reference to the specific activity of the medium. The definition of R used here has the advantage of showing the equilibration of all the phosphate fractions, including the medium, as an ultimate movement of R towards unity.

The product of the relative specific ^{32}P content and the P content of a fraction is proportional to the ^{32}P content, i.e. $R.\mathrm{P}=k.{}^{32}\mathrm{P}$, k being the same as before. Since k is defined as the ratio of P to ^{32}P for the whole system, the sum of the $k.{}^{32}$P contents of all the phosphate fractions should be equal to the total P content of the system—a valuable property for checking the reliability of the ^{32}P estimations. We shall express the quantities of P as μmole/g. cell dry-weight, written μmole/g.

Definition of turnover. Several patterns of phosphate transfer, which might loosely be called turnover, occurred in the present experiments. It will be convenient to consider two of them at this point.

We shall reserve the word turnover to describe the strictly reciprocal exchange of phosphate between two fractions, for it was mainly in this sense that the word was originally used by Schoenheimer (1942). To calculate the rate of turnover of phosphate ($\dot{\mathrm{P}}$) between fractions A and B, considered as a closed system, from the time of half equilibration of ^{32}P ($t_{\frac{1}{2}}$) the following expression (Mitchell, 1953) will be used: $\dot{\mathrm{P}}=(\log_e 2).\mathrm{P}_A.\mathrm{P}_B/(\mathrm{P}_A+\mathrm{P}_B).t_{\frac{1}{2}}$, the symbols P_A and P_B representing the total phosphate contents of fractions A and B.

Precursor-product relationship. In the commonest pattern of transfer resembling turnover, the phosphate passes from a fraction X, through a fraction Y to a fraction Z, the phosphate of fraction Y exhibiting a false turnover. In general, this precursor-product type of pattern may be distinguished from turnover by comparing the observed change of the $k.{}^{32}$P content of fraction Y with the change of the $k.{}^{32}$P content calculated from an integrated form of principle (iii). The integrated form of principle (iii) is simply a continuity equation which may be written:

$$\Delta(k.{}^{32}\mathrm{P}_Y)=\int_{t_1}^{t_2} R_X\,d\mathrm{P}_{X\to Y}-\int_{t_1}^{t_2} R_Y\,d\mathrm{P}_{Y\to Z},$$

or in words: the accumulation of $k.{}^{32}$P in fraction Y during the time (t_2-t_1) is the integral of the R value of fraction X with respect to the flow of P from X to Y minus the integral of the R value of fraction Y with respect to the flow of P from Y to Z. In practice the calculation is made by summing the products of the mean R values and the P fluxes over the series of short time intervals between the collection of cell samples. This method of integration is, of course, approximate, but in the present experiments, the errors introduced were found, by comparison with graphical integration, to be of the same order as the experimental errors.

RESULTS

Cell suspension without added substrate

During the 90 min. of so-called rest in the absence of added substrate (Fig. 1), the R values of the AI, AO and LP rise and that of the mI falls; but the R values of the other fractions do not change significantly. The changes in the R values of the AI, AO, LP and mI might be due either to a reciprocal exchange of phosphate between the fractions or to transfer processes energized by the residual metabolism which occurs in washed suspensions of this organism.

Exchange of inorganic phosphate between cells and medium

Table 1 shows that during the period of rest, neither the P content of the cells nor of the medium change significantly, but a total of 150 μmol. $k.{}^{32}$P/g. are taken up, of which 91 % is accounted for in the AI. Thus, although there is no net transfer, there is an exchange of inorganic phosphate between the cells and medium which is half complete in *c.* 70 min. This gives a rate of turnover of phosphate between the AI and mI of 1·4 μmole/g./min.

Although during the period of rest the rise in the R value of the AI may be attributed to a reciprocal exchange of phosphate with the mI without change of free energy, the rise in the R value of the AO cannot be accounted for in this way. The P content of the AO rises by 16 μmole/g. during the resting period (Table 1), and assuming this phosphate to be supplied via the AI (see below) which has a mean R value of *c.* 0·6 over this period (Fig. 1), the amount of $k.{}^{32}$P accumulating in the AO would be $16 \times 0{\cdot}6 = 9{\cdot}6$ μmole/g. The observed increment of $k.{}^{32}$P in the AO is 14 μmole/g., and since this may be too high by as much as 3 μmole/g. because of contamination of AO with 3 % of AI, the observed increment is within experimental error of the calculated figure. The rise in the R value of the AO in the absence of added substrate may therefore be attributed mainly to the uptake of phosphate into the AO energized by residual metabolism. The data of Table 1 indicate that the rise in the R value of the LP might also be attributed partly to an active uptake of phosphate.

Cell suspension with glucose

During respiration caused by the addition of glucose to the cell suspension, the R values of AI, AO and LP (Fig. 1, continuous lines) approach that of mI more rapidly than during rest, and the R values of PGP, DNA and RNA (Fig. 2, continuous lines) show small but significant increases. The fact that the P content of the DNA remains constant shows that there is no net increase in cell numbers, for it was previously found that the amount of DNA per cell remained constant during growth (Mitchell & Moyle, 1951*a*). Multiplication might, however, occur if accompanied by an equal rate of autolysis. The observed R increments could have resulted from the lysis and growth of only *c.* 1 % of the cells per hour. We cannot therefore tell whether the small changes in the R values of the PGP, DNA and RNA are due to the utilization of the glucose *per se*, or whether they are due to growth of some of the cells on the

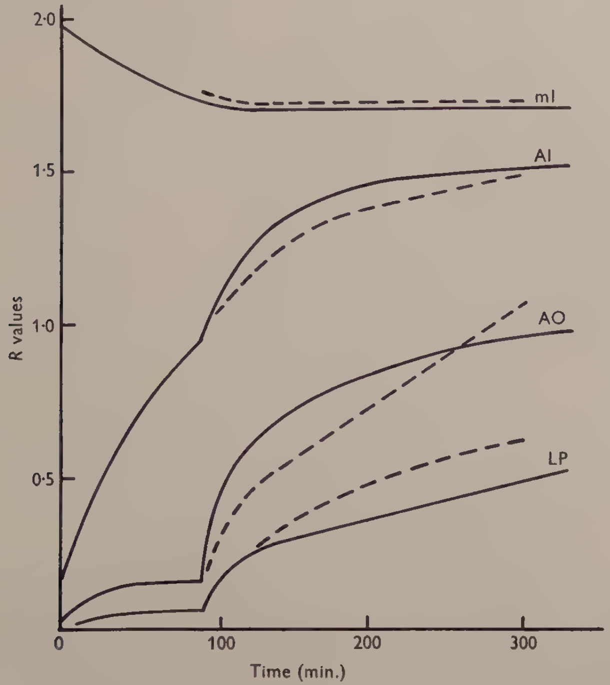


Fig. 1. The time course of the R values of the mI, AI, AO and LP. Cell suspension resting (0 to 90 min.), respiring (continuous lines, 90–330 min.) and growing (broken lines, 90–300 min.). The lines represent the R values taken from Tables 1 and 2.

Table 1. *The* $k.^{32}$P *and* P *contents of the phosphate fractions from* M. pyogenes *during rest and respiration*

		Fractions							
	Cell	mI	AI	AO	LP	DNA	RNA	PGP	Total
Time min.)	dry-wt. (mg./ml.)	µmole $k.^{32}$P and P/g. initial cell dry-wt., written $k.^{32}$P/P=R							
0	10·0	1680/850	28/164	3·1/100	0·3/45	0·1/74	0·2/258	0·5/232	1712/1723
30	10·0	1600/850	89/165	12·7/106	1·4/46	0·2/80	0·6/258	1·7/245	1706/1750
90	10·0	1510/850	164/171	17·2/116	2·6/48	0·3/80	1·3/254	2·2/236	1698/1755
		Glucose added at 90 min. to give 5 % (w/v)*							
113	10·0	1620/950	209/174	70/122	10·4/50	1·6/77	7·7/253	7·9/245	1927/1871
134	10·0	1570/910	242/181	93/142	16·7/60	2·3/75	7·5/245	15·0/273	1946/1886
176	10·0	1440/860	278/200	119/161	20·7/63	2·8/80	10·1/241	21·1/268	1892/1873
220	10·0	1320/770	353/226	155/181	26·1/68	3·3/77	9·8/246	26·3/263	1893/1831
330	10·0	1120/650	413/280	227/235	37/71	3·7/81	9·7/248	40/267	1850/1832

* This solution contained the equivalent of 100 µmole ^{31}P: it affects the total $k.^{32}$P and P correspondingly.

nitrogenous autolysis products of others. On the other hand, there is little doubt that the relatively large increases in the P and $k.^{32}P$ contents of the AI, AO and LP may be attributed to the utilization of glucose.

Uptake of inorganic phosphate. During respiration, the cells take up P at the rate of *c.* 1·4 μmole/g./min. (Table 1) while the *R* value of the mI remains constant (Fig. 1). Thus, the movement of phosphate from the medium to the cells occurs at the same rate as during rest, but the equal and opposite movement of phosphate which occurs during rest is abolished.

Intermediary function of AI. Although, as shown above, there is no turnover of phosphate between the mI and AI during respiration, the $k.^{32}P$ and P contents of the AI/g. increase by 249 and 109 μmole respectively. The *R* value of the mI remains within experimental error of the constant value 1·71 after the sample taken at 90 min. The increment of $k.^{32}P$ which would result simply from the accumulation of phosphate in the AI is therefore $1{\cdot}71 \times 109 = 186$ μmole/g., significantly below the observed value. Another possibility is that the phosphate of all the organic fractions may be drawn through the AI. In this case, since the amount of P entering the AI would be the sum of that entering all the fractions, and the amount leaving would be the same less that accumulating in the AI, and since the *R* values of the mI and AI are known, the $k.^{32}P$ content of the AI may be calculated as described above for the precursor-product system. The value of 242 μmole/g. thus obtained is within experimental error of the observed amount. However, if only half the phosphate of the other fractions were drawn through the AI, we would consider the calculated value, 214 μmole $k.^{32}P$/g. to lie only just significantly below the observed increment. The uptake of ^{32}P into the AI may therefore be explained if the greater part, but not necessarily all, of the phosphate of the other fractions is drawn through the AI. We should point out that the application of principle (iii) is probably legitimate in this case because the AI is unlikely to be unhomogeneous with respect to ^{32}P content.

Accumulation of phosphate in AO. During respiration, the $k.^{32}P$ increment of the AO, calculated from the observed accumulation of P assumed to come from the AI, is 168 while the observed $k.^{32}P$ increment of the AO is 210 μmole/g. Evidently, therefore, a limited amount of turnover occurs between the AI and AO. It should be noted that during respiration, although a small but significant accumulation of P occurs in the LP and PGP, there is no P accumulation in the DNA or RNA. Of the total P taken up by the cells, *c.* 90 % accumulates in the AI and AO.

Cell suspension with glucose and nitrogen source

The addition of glucose and casein hydrolysate to the washed cell suspension causes growth at the rate of *c.* 2 mg./ml./hr. (Table 2). The rate of increase of the P content of the DNA, and therefore the rate of increase in cell numbers, is approximately half that of cell dry-weight, the average weight per cell increasing by a factor of 1·2 during the experiment of Table 2.

During growth, the *R* values of the mI, AI, AO and LP (Fig. 1, broken lines) show practically the same pattern of change as during respiration; but the *R* values of the PGP, RNA and DNA increase more rapidly than during respiration (Fig. 2).

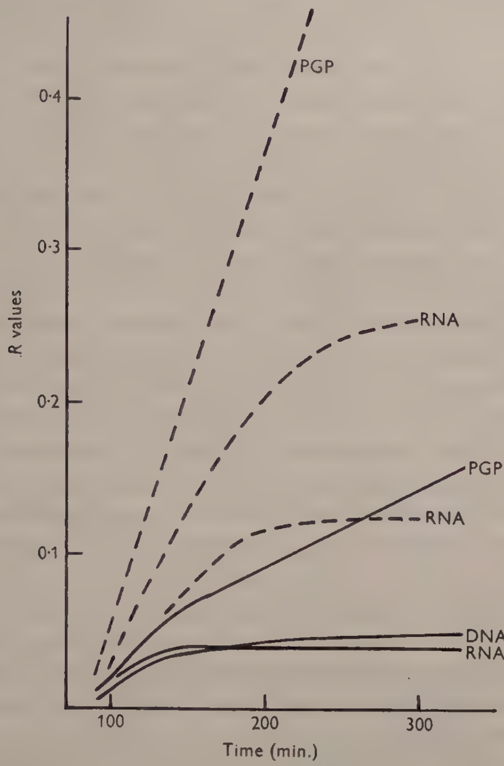


Fig. 2. The time course of the *R* values of the DNA, RNA and PGP. Cell suspension respiring (continuous lines) and growing (broken lines). The lines represent the *R* values taken from Tables 1 and 2.

Table 2. *The k.*^{32}P *and* P *contents of the phosphate fractions during growth*

		Fractions							
	Cell	mI	AI	AO	LP	DNA	RNA	PGP	Total
Time min.)	dry-wt. (mg./ml.)	μmole *k*.^{32}P and P/g. initial cell dry-wt., written *k*.^{32}P/P=*R*							
90	10·0	1730/950	158/165	10·0/87	3·2/62	0·3/102	0/236	7·9/317	1909/1919
Glucose and casein hydrolysate added at 90 min. to give 5 % and 1 % (w/v) respectively*									
130	11·5	1630/980	192/155	46/93	16·8/62	10·4/113	13·4/251	50/341	1959/1995
195	13·5	1540/880	235/174	68/103	29·4/64	24·4/124	37/313	133/367	2067/2025
300	15·4	1290/780	255/171	152/136	44/73	33/131	40/321	250/445	2064/2047

* This solution contained the equivalent of 100 μmole of ^{31}P: it affects the total *k*.^{32}P and P correspondingly.

Uptake of inorganic phosphate. The rate of intake of P by the cells during growth (Table 2) does not differ from that during rest (1·3 as opposed to 1·4 μmole/g./min.) by more than the variation between different batches of resting cells, and the *R* value of the mI remains constant. Presumably the same system causes the uptake of phosphate in growing as in respiring cells.

Intermediary function of AI. The increase in the P content of the AI during growth is relatively small (6 μmole/g.), and, calculating the $k.^{32}P$ increments from the 90 min. sample and taking the *R* value of the mI as 1·72, accounts for only 10·3 μmole/g. of the observed $k.^{32}P$ increment of 97 μmole/g. Assuming, as for the respiring suspension, that the phosphate of all the other fractions is drawn through the AI, a $k.^{32}P$ increment of 131 is obtained, significantly above the observed increment. This corresponds to a total passage of 300 μmole of P and 400 μmole of $k.^{32}P$/g. through the AI, whereas the observed course of the $k.^{32}P$ content of the AI would correspond to *c.* 220 μmole P and 300 μmole $k.^{32}P$/g. The following alternative explanations may account for these observations:

(i) About 70 % of the phosphate of the organic fractions may be drawn through the AI and the remaining 30 % either from the mI or from the unknown carrier compound of the exchange diffusion system (see below). If this were the case, the part drawn through the AO would account for 300 μmole $k.^{32}P$/g., and the part from the mI (or carrier compound) would account for $(300-200)\times 1{\cdot}72=138$, giving a total of 438 μmole $k.^{32}P$/g. This figure is consistent with the 490 μmole found to enter the organic fractions (Table 2), considering that the calculated $k.^{32}P$ contents are small differences between large quantities.

(ii) The whole of the phosphate of the organic fractions may be drawn through the AI while a turnover of phosphate involving the interchange of *c.* 150 μmole phosphate, occurs between the AI and one or more of the organic fractions. At all events, at least 60 % of the phosphate of the organic fractions must be drawn through the AI.

Origin of RNA phosphate. The total amounts of $k.^{32}P$ and P taken up by the RNA during growth are 40 and 85 μmole/g. respectively, giving an apparent mean *R* value for the accumulated phosphate of less than 0·5. It is therefore probable that the bulk of the phosphate of the RNA could not originate from the mI or AI. Assuming that the AO were to supply the phosphate of the RNA, the total $k.^{32}P$ uptake by the RNA (estimated from the *R* values of the AO and the P increments of the RNA) would be 47 μmole/g., in reasonably good agreement with the observed value. It should be emphasized that the AO (consisting mainly of a mixture of nucleotide components) may be assumed to be heterogeneous with respect to ^{32}P content, the *R* values given here being indices of the mean $k.^{32}P/P$ ratio for all the components. Consequently the results of the application of the precursor-product relationship should be accepted with obvious reservations. This does not, however, invalidate the fact that the $k.^{32}P$ content of the RNA behaves as though the components of the AO were incorporated directly into the RNA. Moreover, it is certain that little or no turnover of the phosphate of the RNA can occur with any of the more metabolically active (and therefore ^{32}P rich) fractions during growth.

Origin of DNA phosphate. The total amounts of $k.^{32}P$ and P taken up by the DNA during growth are 33 and 29 μmole/g. respectively, giving an apparent mean R value for the accumulated phosphate of 1·14. It is therefore probable that the bulk of the phosphate of the DNA could not originate from the mI. If the AI were to supply the phosphate of the DNA without turnover, the total $k.{}^{32}P$ uptake by the DNA (estimated as for the RNA) would be 36 μmole/g., within experimental error of the observed value of 33. If, on the other hand, the AO were to supply the phosphate of the DNA, the total $k.{}^{32}P$ uptake would be 16 μmole/g., and to account for the observed value of 33, about one DNA phosphate group would have to exchange with the AO for each new DNA phosphate group taken up.

Intermediary function of AO. During growth, the $k.{}^{32}P$ increment of the AO, calculated for the observed accumulation of P assumed to come from the AI is 67 μmole/g., much more divergent from the observed $k.{}^{32}P$ increment (142 μmole/g.) than was the case during respiration. However, the situation during growth differs from that during respiration in that P accumulates in the RNA, DNA and PGP. Assuming that the P entering the RNA is drawn from the AI, through the AO, the $k.{}^{32}P$ increment of the AO (calculated from the R values of the AI and AO and the increments of the P of the AO and RNA) is 127 μmole/g., within experimental error of the observed value. Alternatively, assuming that the AO is similarly the intermediate for formation of both RNA and DNA, a $k.{}^{32}P$ increment of 147 μmole/g. is obtained, also within experimental error of the observed value. If, however, the P entering all the other fractions were to pass from the AI, through the AO, the $k.{}^{32}P$ increment would be 200 μmole/g., above the experimental value.

During a separation of the AO into free purine, total purine and total pyrimidine (Mitchell & Moyle, 1951*a* and unpublished observations), it was found that at least 80 % of the phosphate of the AO was present in purine and pyrimidine pentose nucleotides, the ease of hydrolysis of the purine nucleotides indicating that they were largely of the purine-3-P type (Caldwell, 1951). The amount of ester phosphate hydrolysed by N-HCl in 10 min. at 100°, after correction for purine nucleotide phosphate was only 10 to 15 % of the total phosphate in different samples, and probably consisted of a mixture of ribose-1-P and nucleotide pyrophosphate phosphorus. No deoxypentose could be detected in the AO by the method of Dische (1930).

We consider it probable, therefore, that the bulk of the AO phosphate consists of the nucleotide precursors of the RNA; that the phosphate of the DNA is drawn from the AI either directly or through intermediates present in small amount in the AO; and that like the RNA phosphate, the DNA phosphate exchanges little if at all with the more metabolically active phosphate fractions. The possibility that the bulk of the phosphate of the DNA could be derived from the RNA or vice versa is absolutely ruled out.

Origin of the PGP phosphate. The total amounts of $k.{}^{32}P$ and P taken up by the PGP during growth are 242 and 128 μmole/g., respectively, giving an apparent mean R value of 1·86 for the accumulated phosphate. It was shown above that the $k.{}^{32}P$ content of the AI indicated that either 30 to 40 % of the phosphate entering the organic fractions during growth is drawn direct from the mI or a turnover involving the interchange of *c.* 150 μmole phosphate/g.

occurs between the AI and one or more of the organic fractions. Of the total P accumulated by the organic fractions c. 55 % is accounted for by uptake without turnover into the AO, RNA and DNA via the AI. The PGP is responsible for c. 40 % of the P uptake.

If the phosphate of the PGP were drawn direct from the mI without turnover, the amount of $k.^{32}P$ entering the PGP would be 204 μmole/g., in agreement with the observed value of 242; whereas, if the phosphate were similarly drawn from the AI, the amount of $k.^{32}P$ entering the PGP would be 153. Thus, the PGP phosphate might be drawn either direct from the mI (or the carrier compound of the exchange diffusion system) without significant turnover, or via the AI with a turnover corresponding to the exchange of one phosphate group for every two phosphate groups entering the PGP.

Origin of the LP phosphate. The total amounts of $k.^{32}P$ and P taken up by the LP during growth are 41 and 11 μmole/g., respectively, giving an apparent mean R value of 3·7 for the accumulated phosphate. The LP accounts for less than 5 % of the total P uptake by the organic fractions, and, as with the PGP, it is not possible to determine whether the phosphate is taken up directly from the mI or via the AI. It is, however, evident that the LP shows the highest unequivocal turnover whether it be with the mI or AI.

DISCUSSION

The exchange of 'inorganic phosphate' between resting cells and their suspension medium might be attributed either to exchange adsorption, proposed by Roberts & Roberts (1950), or to a type of mechanism first described by Lundegårdh (1945) and later named exchange diffusion by Ussing (1947). The importance of making a decision between these hypotheses has led one of us to study the characteristics of the exchange reaction in detail. The first results of this study (Mitchell, 1953) show that the osmotic barrier of *M. pyogenes* is impermeable to phosphate ions and that the movement of phosphate groups across the osmotic barrier is regulated by an exchange diffusion system.

Since the rate of intake of inorganic phosphate during respiration is the same as the rate of exchange across the osmotic barrier during rest, we suggest that the same system is responsible for both processes, and that during respiration a source of free energy is made available to do the necessary osmotic work.

Labaw, Mosley & Wyckoff (1950) and Roberts & Roberts (1950) found that very little ^{32}P is lost from growing suspensions of *Bacterium coli* when transferred from a medium containing ^{32}P-labelled phosphate to a ^{32}P-free medium. Caldwell, Mackor & Hinshelwood (1950) made a similar observation on *Aerobacter aerogenes* and concluded that '...while the nucleic acid is actually participating in the synthesis of protein, little of its phosphorus undergoes exchange...'. We have shown, however, that in *M. pyogenes*, during respiration or growth, phosphate groups pass only inwards through the osmotic barrier. Hence, the demonstration that phosphate (^{32}P) fails to pass out of an organism into the medium during growth cannot be taken to show that the phosphate of the RNA or DNA does not turn over with other phosphate compounds inside the cells. Nevertheless, we have obtained some experimental support for the view that during growth the RNA and DNA phosphate exchanges little if at all in *M. pyogenes*.

According to Spiegelman and his collaborators (Juni *et al.* 1948), a turnover of phosphate occurs in the nucleic acid of baker's yeast during growth; and phosphate turnover has also been reported for the RNA and DNA of *Proteus vulgaris* by Levy, Scutch & Schade (1949). The meaning attached to the word turnover by these authors appears, however, to be rather a loose one. An examination of the experimental data indicates that neither in baker's yeast nor in *P. vulgaris* has a significant reciprocal exchange of phosphate between the nucleic acids and any other phosphate fraction been demonstrated during growth.

The DNA of *M. pyogenes* resembles that of mammalian tissue cells in exhibiting little or no phosphate turnover; whereas the lack of RNA phosphate turnover in *M. pyogenes* is in contradistinction to the behaviour reported for the RNA of mammalian tissue cells (e.g. Brues, Tracy & Cohn, 1944; Furst, Roll & Brown, 1950).

A point which does not generally seem to be recognized when considering low turnover values is that a cell suspension differs qualitatively from a single cell because it may exhibit a false type of turnover due to simultaneous lysis and regeneration of cells. A similar false turnover may also be given by tissues in which the cells or their sub-units absorb precursor substances and release their products.

In stating that the turnover of the phosphate of the RNA and DNA is not a necessary accompaniment of the processes of synthesis described as growth, we do not wish to imply that the nucleic acids take no part in the synthetic processes. On the contrary, the stability of the nucleic acids in the cells would seem to be in accord with the controlling function which has often been attributed to them.

The formation of DNA from RNA, originally considered by Brachet (1933) and shown to be unlikely by Schmidt, Hecht & Thannhauser (1948) and by Villee, Lowens, Gordon, Leonard & Rich (1949) has been considered again by Caldwell & Hinshelwood (1950, 1951). The evidence of the present work is against transfer of phosphate between the bulk of the RNA and DNA either directly or indirectly. The observations that phage DNA synthesis in *Bact. coli* occurs at the expense of the DNA phosphate (Koch, Putnam & Evans, 1952), but not at the expense of the host RNA phosphate (Cohen, 1951), likewise shows that in phage infected *Bact. coli* a transfer of phosphate from the RNA to DNA does not occur. We have also been informed by J. D. Watson that in unpublished work employing ^{32}P, he has found no transfer of phosphate either from RNA to DNA or vice versa in normal growing cultures of *Bact. coli*.

Our conclusions as to the pathway of the phosphate which goes to form RNA and DNA during growth of *M. pyogenes* can best be described diagrammatically (see Fig. 3).

The phosphate required for the formation of the glycerophosphoprotein complex and the phospholipid originates from the inorganic phosphate either of the external medium or of the cell interior. Had the PGP or LP been situated only inside the osmotic barrier, a decision between these alternatives would have been possible. Unfortunately, however, both the PGP and LP form a substantial part of the cell envelope (Mitchell & Moyle, 1951*b*) and are probably accessible to both internal and external media.

It was thought at the outset of the present work that the PGP, being the predominant phosphate ester of the cell envelope, might be the intermediate by which phosphate was transferred from the medium to the cell interior. However, the PGP as a whole cannot be the postulated intermediate because it does not exhibit a significant turnover in resting cell suspensions in which a rapid exchange of inorganic phosphate occurs. It is also evident that the PGP is not involved in the phosphate transfer reactions associated with respiration since the PGP phosphate exhibits little or no true turnover in respiring suspensions. During growth, however, while the number of cells (DNA phosphate) increased by a factor of 1·29 the cell dry-weight increased by a factor of 1·55 and the PGP by a factor of 1·41. Hence the average weight per cell increased by a factor of 1·20 and, as the cells do not change their shape or density appreciably during growth, the average area per cell must have increased by a factor of $(1{\cdot}20)^{\frac{2}{3}}=1{\cdot}13$. The total cell area must therefore have increased by a factor of $1{\cdot}13\times1{\cdot}29=1{\cdot}46$—within the experimental error of the observed PGP increase. Thus, the PGP production and cell area expansion occur at the same rate in accordance with the fact that the mechanically resistant part of the cell envelope of *M. pyogenes* is composed of PGP.

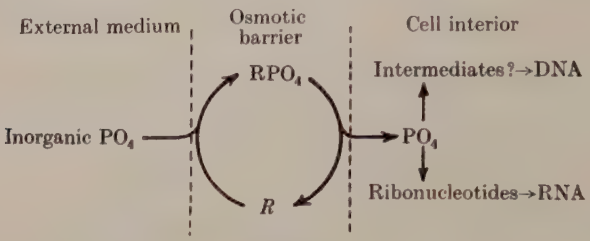


Fig. 3

It might be expected that the closely associated PGP and LP would have a common phosphate metabolism, especially as the phosphate is present in both as glycerol esters. But, while the PGP and LP may have a common glycerophosphate precursor, the accessibility of the phosphate of the LP for exchange is evidently much greater than that of the PGP.

As a whole, our observations lead to the conclusion that most of the organic phosphate compounds of the cells of *M. pyogenes* do not exchange their phosphate groups at a rate comparable to that of phosphorylation during growth. In other words, the enzymes catalysing the formation of the phosphate compounds do not catalyse their interchange at a comparable rate. It follows that the reactions causing the synthesis of the organic phosphate compounds do not normally take place near equilibrium.

We are indebted to Dr J. Boursnell for allowing us to use his counting machinery to estimate ^{32}P. One of us (J.M.M.) wishes to acknowledge the receipt of a grant from the Medical Research Council in support of this work.

REFERENCES

Boursnell, J. C., Coombs, R. R. & Rizk, V. (1953). Studies with marked antisera. *Biochem. J.* In the press.

Brachet, J. (1933). Recherches sur le synthèse de l'acide thymonucléique pendant le développement de l'œuf d'oursin. *Arch. Biol., Paris*, **44**, 519.

Brues, A. M., Tracey, M. M. & Cohn, W. E. (1944). Nucleic acids of rat liver and hepatoma: their metabolic turnover in relation to growth. *J. biol. Chem.* **155**, 619.

Caldwell, P. C. (1951). The nature of the phosphorus compounds present in cells of *Bact. lactis aerogenes*. *J. chem. Soc.* p. 166.

Caldwell, P. C. & Hinshelwood, C. (1950). Some considerations on autosynthesis in bacteria. *J. chem. Soc.* p. 3156.

Caldwell, P. C. & Hinshelwood, C. (1951). The phosphorus metabolism of *Bact. lactis aerogenes*. *J. chem. Soc.* p. 158.

Caldwell, P. C., Mackor, E. L. & Hinshelwood, C. (1950). The ribose nucleic acid content and cell growth of *Bact. lactis aerogenes*. *J. chem. Soc.* p. 3151.

Cohen, S. S. (1951). Biochemical studies on multiplication of bacterial viruses. *Fed. Proc.* **10**, 585.

Dische, Z. (1930). Über einige neue characteristische Farbreaktionen der Thymonucleinsäure und eine Mikromethode zur Bestimmung derselben in tierischen Organen mit Hilfe dieser Reaktionen. *Mikrochemie*, **8**, 4.

Fiske, C. H. & Subbarow, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375.

Furst, S. S., Roll, P. M. & Brown, G. B. (1950). On the renewal of the purines of the desoxypentose and pentose nucleic acids. *J. biol. Chem.* **183**, 251.

Juni, E., Kamen, M. D., Reiner, J. M. & Spiegelman, S. (1948). Turnover and distribution of phosphate compounds in yeast metabolism. *Arch. Biochem.* **18**, 387.

Koch, A. L., Putnam, F. W. & Evans, E. A. (1952). Biochemical studies of virus reproduction. VIII. Purine metabolism. *J. biol. Chem.* **197**, 113.

Labaw, L. W., Mosley, V. M. & Wyckoff, R. W. G. (1950). Radioactive studies of the phosphorus metabolism of *Escherichia coli*. *J. Bact.* **59**, 251.

Levy, H. B., Skutch, E. T. & Schade, A. L. (1949). Effect of cobalt on the phosphorus turnover rate in the nucleic acids of *Proteus vulgaris*. *Arch. Biochem.* **24**, 206.

Lundegårdh, H. (1945). Absorption, transport and exudation of inorganic ions by the roots. *Ark. Bot.* **32** A, 12, 1.

Mitchell, P. (1949). A new technique for stirred aerated culture. *Nature, Lond.* **164**, 846.

Mitchell, P. (1953). Transport of phosphate across the surface of *Micrococcus pyogenes*: nature of the cell 'inorganic phosphate'. *J. gen. Microbiol.* **9**, 273.

Mitchell, P. & Moyle, J. (1951*a*). Relationships between cell growth, surface properties and nucleic acid production in normal and penicillin-treated *Micrococcus pyogenes*. *J. gen. Microbiol.* **5**, 421.

Mitchell, P. & Moyle, J. (1951*b*). The glycerophospho-protein complex envelope of *Micrococcus pyogenes*. *J. gen. Microbiol.* **5**, 981.

Nelson, V. (1944). A photometric adaptation of the somogyi method for the determination of glucose. *J. biol. Chem.* **153**, 375.

Roberts, R. B. & Roberts, I. Z. (1950). Potassium metabolism in *Escherichia coli*. III. Interrelationship of potassium and phosphorus metabolism. *J. cell. comp. Physiol.* **36**, 15.

Schmidt, G., Hecht, L. & Thannhauser, S. J. (1948). The behaviour of the nucleic acids during the early development of the sea urchin egg. *J. gen. Physiol.* **31**, 203.

Schmidt, G. & Thannhauser, S. J. (1945). A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. *J. biol. Chem.* **161**, 83.

Schoenheimer, R. (1942). *The dynamic state of the body constituents.* Harvard Univ. Press, Cambridge, Mass.

Ussing, H. H. (1947). Interpretation of the exchange of radio-sodium in isolated muscle. *Nature, Lond.* **160**, 262.

Villee, C. A., Lowens, M., Gordon, M., Leonard, E. & Rich, A. (1949). The incorporation of ^{32}P into the nucleoproteins and phosphoproteins of the developing sea urchin embryo. *J. cell. comp. Physiol.* **33**, 93.

(*Received* 28 *March* 1953)

MITCHELL, P. (1953). *J. gen. Microbiol.* 9, 273–287.

Transport of Phosphate across the Surface of *Micrococcus pyogenes*: Nature of the Cell 'Inorganic Phosphate'

BY P. MITCHELL

Department of Biochemistry, University of Cambridge

SUMMARY: An osmotic barrier, impermeable to inorganic phosphate ions, is present near the external surface of *Micrococcus pyogenes* var. *aureus* (strain Duncan). The barrier encloses a volume of 1·66 ± 0·07 ml./g. dry-wt. cells, within which the inorganic phosphate of the cell is confined at a concentration of *c.* 0·1 M. The inorganic phosphate of the internal and external media exhibit a rapid reciprocal exchange, sensitive to traces of heavy metals. The existence of the osmotic barrier and the characteristics of the phosphate exchange are not in accord with an exchange-adsorption hypothesis; they are in accord with an exchange-diffusion hypothesis, according to which phosphate is shuttled across the osmotic barrier by carrier molecules. There is no experimental justification for supposing the acid-soluble inorganic phosphate of *M. pyogenes* to exist as an acid-labile compound.

Considerable amounts of inorganic orthophosphate can be extracted from most bacteria and tissue cells with cold trichloroacetic acid; but it has not been established whether this inorganic phosphate is normally in the free state and held within the cells by an acid-sensitive osmotic barrier, or whether it is held in an acid-labile compound forming part of the cell framework. Attention has been directed towards the latter view by Green and his collaborators (e.g. Green, Atchley, Nordman & Tepley, 1949) working on the 'gel phosphate' of the cyclophorase system of mammalian tissues, by Harman (1950) working on mammalian heart muscle mitochondria, by Spiegelman's group (Kamen & Spiegelman, 1948) working on baker's yeast and by Roberts & Roberts (1950) working on *Bacterium coli*. According to Roberts & Wolfe (1952), *Bact. coli* is permeable, not only to inorganic phosphate ions, but even to sugar phosphates. It is therefore necessary to postulate that the acid-soluble inorganic phosphate of *Bact. coli* normally exists as an acid-labile compound which cannot diffuse from the cells.

In resting suspensions of *Micrococcus pyogenes* Mitchell & Moyle (1953) found, as in *Bact. coli* (Roberts & Roberts, 1950), that a reciprocal exchange occurs between the acid-soluble inorganic phosphate of the cells and the inorganic phosphate of the suspension medium. This exchange might be attributed either to exchange-adsorption, as proposed by Roberts & Roberts (1950) or to a type of mechanism first suggested by Lundegårdh (1945) and later described more exactly and named exchange-diffusion by Ussing (1947). The work described in the present paper was designed to eliminate one or other of the exchange hypotheses in the case of *Micrococcus pyogenes* var. *aureus* (strain Duncan). We shall formulate the hypotheses as follows.

Exchange-adsorption hypothesis. The acid-soluble inorganic phosphate is normally held in the cells as a labile compound, freely accessible to the inorganic phosphate of the medium. The degree of dissociation of the compound into free phosphate and combining group is very small so that the total amount of bound (acid-soluble) phosphate is practically independent of the concentration of inorganic phosphate in the medium. The reaction between the free phosphate and combining group is reversible, so that a reciprocal exchange of 'inorganic phosphate' between the cells and medium may occur.

Exchange-diffusion hypothesis. The acid-soluble inorganic phosphate exists in the cells as inorganic phosphate, separated from the inorganic phosphate of the medium by an osmotic barrier which is impermeable to phosphate ions but contains carrier groups. The carrier groups cannot leave the barrier, but, owing to thermal agitation, alternately come into contact with the solution on either side, where exchanges may occur between the carrier-phosphate groups and free phosphate ions. For every phosphate group which passes one way across the barrier on a carrier group, the same or another phosphate group must pass the other way when the carrier group returns.

Two kinds of experimental approach towards a decision between the exchange hypotheses suggest themselves.

Permeability measurement. The part of the volume of a cell suspension not accessible to externally added phosphate should be equal to the effective volume of the cells bounded by the hypothetical osmotic barrier. If such a barrier were to exist near the external surface of *M. pyogenes*, the effective cell volume should be approximately equal to the volume of a close-packed centrifuged pad less 26 %, the interspace volume for close-packed spheres (Conway & Downey, 1950). On the other hand, if there were no osmotic barrier, or if the barrier were broken, the effective cell volume should be approximately equal to the apparent specific volume of the major components of the cell, of the order of 0·8 ml./g. dry-wt. cells.

Inhibition of exchange. If the exchange-adsorption hypothesis were correct, the acid-soluble inorganic phosphate of the cell would be held by a fixed number of combining groups corresponding in number to the acid-soluble inorganic phosphate molecules. The inhibition of the exchange of the phosphate on a combining group would not be expected to occur unless the inhibitor molecule were close to the combining group. Hence, unless action at a distance were postulated, the number of molecules of a low molecular weight inhibitor required to retard the exchange reaction would have to be of the same order as the number of molecules of acid-soluble inorganic phosphate. If, on the other hand, the exchange-diffusion hypothesis were correct, the exchange reaction would be mediated by a relatively small number of carrier groups in the osmotic barrier. In this case, an inhibitor might retard the exchange reaction when the number of molecules present was similar to the number of carrier groups and therefore small in comparison to the number of molecules of acid-soluble inorganic phosphate.

It was clear at the outset of this work that permeability measurement, by showing the presence or absence of an osmotic barrier for phosphate, would show whether the final system for regulation of exchange was of the exchange-adsorption or of the exchange-diffusion type. The inhibitor studies, however, involved a considerable risk; for, although they might disprove the exchange-adsorption hypothesis (depending upon the chance of finding a sufficiently potent inhibitor for the exchange-diffusion system), they would not give positive evidence in favour of exchange-adsorption. Permeability measurements were therefore made first, and when these indicated the occurrence of exchange-diffusion it was considered worth while to begin the inhibitor studies.

METHODS

Growth and preparation of organism. Cultures of *M. pyogenes* were grown as previously described (Mitchell & Moyle, 1953). The cells were harvested at a concentration equivalent to 2·5 mg. dry-wt./ml., washed three times in distilled water and suspended at a concentration equivalent to *c.* 200 mg. dry-wt./ml. for the permeability measurements and *c.* 100 mg./ml. for the exchange measurements. The pH of the suspensions, measured with a glass electrode, was *c.* 5·5 after washing: it was adjusted to the appropriate value with dilute HCl or NaOH immediately before each experiment, the degree of dilution being recorded to correct the dry-weight concentration. The suspensions, stored at 2°, maintained their phosphate-exchanging activity for up to 48 hr. After this, the cells deteriorated rapidly and much of their acid-soluble inorganic phosphate escaped. In the present work, suspensions not more than 12 hr. old were used.

Estimation of P *and* ^{32}P. The methods used were as previously described (Mitchell & Moyle, 1953).

Estimation of dry-weight. The approximate concentrations of the washed cell suspensions in distilled water were adjusted turbidimetrically. The exact dry-weight concentrations were subsequently measured by drying samples containing *c.* 200 mg. to constant weight at 105° and weighing to 0·5 mg. The suspension densities subsequently described in mg./ml. refer to mg. dry-wt. cells/ml.

Permeability measurements

Normal cells. It was advisable to determine the external phosphate space in the presence of a relatively large concentration of salt to minimize the adsorption of phosphate on the cell surfaces and to control the osmotic and ionic condition of the medium. Sodium chloride and phosphate were therefore added in amounts which would give final concentrations in the external water space of about 100 mM and 5 mM, respectively. Preliminary experiments showed that in the concentrated cell suspension containing *c.* 200 mg./ml., the water space accessible to externally added phosphate was *c.* 0·5 ml./ml. suspension. Two ml. samples of the concentrated suspension, adjusted to pH 7 were pipetted into test-tubes 7 cm. in length, of total capacity 2·5 ml. To this suspension, 0·1 ml. M-NaCl alone (control) or containing 0·025 M-Na_2HPO_4 + 0·025 M-NaH_2PO_4 (test) was added. The suspensions were rapidly mixed with a fine glass rod and centrifuged at 3000 *g* for 1 hr. at 2°, either immediately or after standing at 25° for 30 min. The concentrations of phosphate were estimated in duplicate 0·1 ml. samples of the supernatants and called [control P] and [test P]. To compensate for systematic volumetric errors, 0·1 ml. of the M-NaCl containing 0·05 M-phosphate was pipetted into 1 ml. distilled water and the phosphate concentration was estimated in 0·1 ml. samples of this dilution exactly as for the supernatants and called [standard P]. The effective cell volume (V ml.) was calculated from the equation:

$$\frac{[\text{standard P}]}{[\text{test P}]-[\text{control P}]}=\frac{2{\cdot}1-V}{1{\cdot}1}. \tag{1}$$

The total supernatant volumes were estimated in graduated pipettes, and the volumes of the centrifuged cell pads were obtained by difference. Pipettes with extremely fine tips were used so that the supernatant liquid could be quantitatively drawn into them with a thick-walled rubber teat.

Damaged cells. The acid-soluble inorganic phosphate is liberated from suspensions of *M. pyogenes* within 20 min. by 5 % trichloroacetic acid at 2°. The experiment described in Fig. 1 showed that the acid-soluble inorganic phosphate is also quantitatively liberated within 20 min. at 15° and at pH 7 by concentrations of *n*-butanol exceeding 4 % (w/v). The external phosphate space and effective cell volume in suspensions of cells damaged by trichloroacetic acid or butanol were determined as follows. Two ml. samples of the concentrated suspension of normal cells were pipetted into test-tubes 10 cm. in length of total capacity 6 ml. The suspensions were centrifuged at 3000 g for 1 hr. and 0·5 ml. of the supernatants was discarded. Butanol (0·1 ml.) or trichloroacetic acid (0·15 ml. of a 50 % (w/v) solution) was now added to pairs of tubes and stirred rapidly into the pads of cells with a fine glass rod. After 20 min., one of each pair was appropriately diluted with 3 ml. of 5 % (w/v) butanol or trichloroacetic acid, and the undiluted and diluted suspensions were centrifuged at 3000 g for 1 hr. The volumes of the supernatants from the

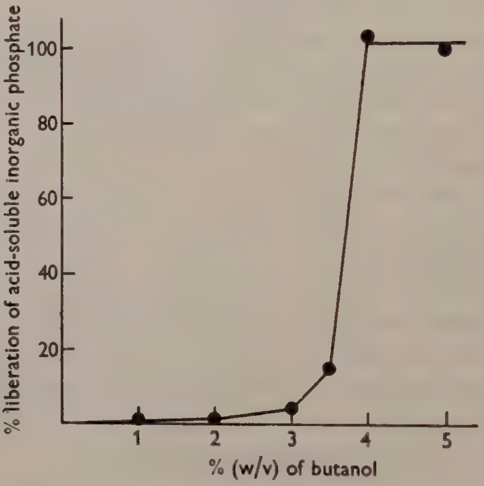


Fig. 1. The percentage of the acid-soluble inorganic phosphate of a 20 mg./ml. cell suspension liberated by *n*-butanol in 20 min. at 15° at pH 7.

undiluted suspensions were measured so that the contraction of the cell pads on treatment with butanol or trichloroacetic acid could be obtained by difference. The concentration of phosphate was estimated in the supernatants from the undiluted ([undil. P]) and diluted ([dil. P]) suspensions, using 0·2 ml. samples, suitably diluted. The effective volumes of the cells in trichloroacetic acid (v_A ml.) and in butanol (v_B ml.) were calculated from the equations:

$$\frac{[\text{undil. P}]}{[\text{dil. P}]}=\frac{4{\cdot}65-v_A}{1{\cdot}65-v_A}\quad\text{for trichloroacetic acid.}\tag{2}$$

$$\frac{[\text{undil. P}]}{[\text{dil. P}]}=\frac{4{\cdot}60-v_B}{1{\cdot}60-v_B}\quad\text{for butanol.}\tag{3}$$

Previous experience indicated that the standard deviation of the phosphate and volumetric measurements was *c.* ±1 %. The values of V and v are given by differences between experimentally measured values about twice and five times as large, respectively. It was anticipated, therefore, that it should be possible to measure V to within about ±2 % and v to within about ±5 %.

Exchange measurements

The rate of exchange of inorganic phosphate across the cell surface can be determined by labelling the phosphate of the medium or of the cells with ^{32}P and subsequently observing the rate of mixing of ^{31}P and ^{32}P.

Theoretical. Assuming the system regulating the exchange of inorganic phosphate to be of the exchange-diffusion type, a simple relationship can be shown to exist between the effective rate of shuttling ($\dot{\mathrm{P}}$) of phosphate by the carrier compound in the osmotic barrier, the total amounts of inorganic phosphate in the internal ($\mathrm{P_I}$) and external ($\mathrm{P_E}$) media and the rate of change of the relative specific activity of the phosphate on either side of the barrier. We shall suppose that the probability of carriage across the barrier of a molecule of phosphate containing ^{32}P is the same as the proportion of the total number of inorganic phosphate molecules which contain ^{32}P, namely $^{32}\mathrm{P}/\mathrm{P}$, on the side of the barrier from which the phosphate is being carried. Hence, the net rate of transfer of ^{32}P into the cells may be written

$$\frac{d\,{}^{32}\mathrm{P_I}}{dt}=\dot{\mathrm{P}}\left(\frac{^{32}\mathrm{P_E}}{\mathrm{P_E}}-\frac{^{32}\mathrm{P_I}}{\mathrm{P_I}}\right).\tag{4}$$

In the present experiments, it will be shown that the amounts of total inorganic phosphate and phosphate containing ^{32}P were constant so that

$$\mathrm{P_I}+\mathrm{P_E}=k({}^{32}\mathrm{P_I}+{}^{32}\mathrm{P_E}),\quad k\ \text{being a constant.}\tag{5}$$

The relative specific activity R, is defined by

$$R=k\,.{}^{32}\mathrm{P}/\mathrm{P}.\tag{6}$$

Eliminating either $^{32}\mathrm{P_E}$ or $^{32}\mathrm{P_I}$ and assuming $\mathrm{P_E}$ and $\mathrm{P_I}$ to be constants, equation 4 can be integrated from time t_1 to t_2, giving

$$\dot{\mathrm{P}}=\frac{1}{t_2-t_1}\cdot\frac{\mathrm{P_E}\,.\,\mathrm{P_I}}{\mathrm{P_E}+\mathrm{P_I}}\ln\left(\frac{1-R_{t_1}}{1-R_{t_2}}\right)_{\mathrm{I\ or\ E}}.\tag{7}$$

The R values at times t_1 and t_2 inside the natural logarithm (ln) of equation (7) both refer either to the internal or to the external medium. It was convenient to express t in minutes and P in μmole/g. cell dry-weight (μmole/g.). The units of $\dot{\mathrm{P}}$ are therefore μmole/g./min.

It should be understood that equation (7) describes the strictly reciprocal exchange of a component between two systems in which the amount of the component remains constant: it is independent of the nature of the exchange mechanism. Similar equations have been used extensively in the past, the most recent derivation being by Krebs, Eggleston & Terner (1951) who refer to the earlier literature.

Experimental. In most experiments, the external medium was marked with ^{32}P and the decrease in R_E and the increase in R_I were determined during a time interval measured from the moment of adding the ^{32}P. It was technically most convenient to determine the ^{32}P content of the cells (from which the R_I value was obtained) in the presence of a considerable proportion of the phosphate of the medium. It was therefore necessary to work with external concentrations of phosphate low enough to make P_I of comparable magnitude to P_E in cell suspensions of reasonably low concentration. In such suspensions, measurements could be made equally well when the inorganic phosphate of the cells was initially marked with ^{32}P and the value of R_E was determined. The technique described below was found to give satisfactory results.

Basic technique. Into test tubes 10 cm. long and total capacity 6 ml., standard solutions were measured to give final concentrations (in 5 ml.) of 0·1 M-NaCl and 2 mM-sodium phosphates containing 1 μC. of ^{32}P/ml. Solutions of substances expected to inhibit or stimulate phosphate exchange were added to give the required final concentration, and dilute HCl or NaOH were added to give the required final pH value, measured in duplicate samples with a glass electrode. The volume was made up to 4 ml. with distilled water. After temperature equilibration at 15° or 25°, 1 ml. of washed cell suspension (*c.* 100 mg./ml.) was rapidly mixed with 4 ml. of the medium and the resulting suspension was kept at constant temperature for a measured time. It was then centrifuged at 3000 g for 10 min. and 3 ml. of the supernatant medium were withdrawn. Three ml. of distilled water were added to the remaining 2 ml., and the cells redispersed. Duplicate 0·1 ml. samples of the supernatant medium and redispersed suspension were taken to estimate the concentrations of ^{32}P (M and N counts/ml. respectively), and duplicate 0·2 ml. samples of the supernatant medium were taken to estimate P (r μmole/ml.). The total amounts of ^{32}P and P were calculated as counts or μmole in Z ml. (*c.* 50 ml.) of the suspension at the concentration of *c.* 20 mg./ml., representing counts or μmole/g. cells. For each batch of cells the value of Z was determined gravimetrically. The external phosphate space in the suspension was 0·95 ml./ml. (see below). Hence, $^{32}\mathrm{P}_E = M.Z/0{\cdot}95$ and $\mathrm{P}_E = r.Z/0{\cdot}95$. After withdrawing 3 ml. of the medium from 5 ml. of centrifuged suspension, only 1·75 ml. of the external phosphate space remained, and this was diluted to 4·75 ml. when the suspension was redispersed. Hence, $^{32}\mathrm{P}_I = Z.(N - {}^{32}\mathrm{P}_E.1{\cdot}75/4{\cdot}75)$.

To measure the inorganic P content of the cells, 1 ml. of the washed cell suspension was added to each of two tubes, one containing 4 ml. distilled water (blank) and the other 4 ml. of 6·25% (w/v) trichloroacetic acid. After 20 min. at 2°, the suspensions were centrifuged, and duplicate 0·2 ml. samples were taken to estimate the concentration of phosphate in the blank supernatant (b μmole/ml.) and in the trichloroacetic acid extract (a μmole/ml.). The external phosphate space was 0·98 ml./ml. in the trichloroacetic acid-treated suspension (see below). Hence, $P_I = Z.(a-b)/0{\cdot}98$. In the present work, b never exceeded 5% of a.

Having obtained P_E, P_I, $^{32}P_E$ and $^{32}P_I$, the values of R_E and R_I could be found, using equations (5) and (6). For any single experiment, R_E and R_I are not, of course, independent variables, and substitution of either R_E or R_I into equation (7) will give the same value for P. In practice, however, several suspensions were handled together so that mean values for $^{32}P_E + {}^{32}P_I$ and $P_E + P_I$ could be obtained. These values were occasionally used to calculate R_E or R_I independently in a group of experiments where a P value was obviously false or was lost by an accident. But, in general, the mean values were employed only as a check on the reliability of individual measurements.

Separation of organic and inorganic components of P_I. In order to find the proportions of the phosphate entering the organic and inorganic acid-soluble fractions during exchange, the basic technique was varied in the following way. At the end of the time allowed for exchange, the 5 ml. of suspension were centrifuged, and 3 ml. of the medium were withdrawn as before. The cells were redispersed in the remaining 2 ml. and 0·4 ml. of 30% (w/v) trichloroacetic acid was added. The suspension was centrifuged after 20 min. at 2°, and the total P, inorganic P and ^{32}P contents of the supernatant were determined using 0·1 ml. samples. To 1 ml. of the supernatant, 0·05 ml. 20 N-ammonium hydroxide and 0·05 ml. magnesia mixture were added, and the inorganic phosphate was allowed to precipitate at 2° overnight. The precipitate was centrifuged and 0·1 ml. samples of the supernatant were taken for estimation of the organic P and organic ^{32}P contents. The mutual contamination of the organic and inorganic P separated by this method was not greater than 3%. The values of P_E, P_I, $^{32}P_E$ and $^{32}P_I$ were calculated by applying principles similar to those of the basic technique, the main difference being that P_E and $^{32}P_E$ were now divided into inorganic and organic fractions.

Reversal of movement of ^{32}P. The technique used for measuring the phosphate exchange by the passage of ^{32}P from cells to medium was essentially the same as the basic technique. It differed in that the cells were first incubated for 30 min. at 25° in 0·1 M-NaCl at pH 7, at a dry-weight concentration of 20 mg./ml., with 1 μC. of ^{32}P-marked phosphate/ml. containing only *c.* 10^{-5} M-carrier phosphate. The cell suspension was centrifuged, washed once in distilled water, and resuspended in distilled water at a concentration of *c.* 100 mg./ml. This suspension was used as in the basic technique, the ^{32}P of the medium being omitted.

RESULTS

Permeability

Effective cell volumes. The volumes of *M. pyogenes* not penetrable by inorganic phosphate before and after injury by 5% trichloroacetic acid or butanol are shown in Table 1. Whereas, before injury, the mean volume was 2·42 ± 0·05 ml./g., after injury it decreased to 0·76 ± 0·07 ml./g. There must therefore be an internal space in intact cells which is not accessible to externally added phosphate. It was conceivable that this internal space might normally be inaccessible to free phosphate ions because of electrostatic repulsion by negative groups within a gel structure, and that trichloroacetic acid might make the space available by discharging the gel. This cannot, however, be the case because butanol at pH 7 was as effective as trichloroacetic acid in allowing penetration of inorganic phosphate.

Table 1. *Effective phosphate-impenetrable volumes of centrifuged cells of* Micrococcus pyogenes

	Normal cells		Cells damaged by 5% (w/v)	
	Initial	30 min. later	Trichloroacetic acid	*n*-Butanol
	Phosphate-impenetrable volumes (ml./g. dry-wt. cells)			
	2·45	2·44	0·68	—
	2·33	2·39	0·62	—
	2·47	—	0·80	0·83
	2·45	—	—	0·71
	2·40	—	—	0·89
Means	2·42 ± 0·05		0·76 ± 0·07	

When the cells were damaged by trichloroacetic acid or butanol, practically none of the protein or nucleic acid escaped, but 18% of the dry weight was lost as soluble components of relatively small molecular weight, which could readily pass through cellophan dialysis tubing. The effective volume of the cell material which centrifuged down after damage was therefore 0·76 ± 0·07 ml./0·82 g. = 0·93 ± 0·09 ml./g. This value is within the range expected for the apparent specific volume of the cell components.

The volumes of the close-packed pads of cells are shown in Table 2. The volume of the cell pad decreased by 26·9 ± 1·4% after damage by trichloro-

Table 2. *Close-packed cell pad volumes*

		Cells damaged by 5% (w/v)		
	Normal cells	Trichloroacetic acid	*n*-Butanol	
	Cell volumes (ml./g. dry-wt. cells)			% contraction
	3·60	2·68	—	25·6
	3·60	2·58	2·58	28·3
	3·62	—	2·70	25·4
Means	3·61 ± 0·01	2·63 ± 0·06		26·9 ± 1·4

acetic acid or butanol. This agrees well with the figure of 26% calculated by Conway & Downey (1950) for the interspace volume for close-packed spheres. The inference is that when the osmotic barrier is damaged, the cells lose their internal turgor pressure and are then capable of distortion into dodecahedra with zero interspace. If the external surfaces of the osmotic barriers were to make contact, the volume of the close-packed damaged cells ($2{\cdot}63 \pm 0{\cdot}06$ ml.) should be equal to the phosphate impenetrable volume ($2{\cdot}42 \pm 0{\cdot}05$ ml.). The difference $0{\cdot}21 \pm 0{\cdot}06$ ml. (*c.* 9% of the cell volume) represents the amount of water lying between the osmotic barriers of the cells. The cells are about 0·7 μ. in diameter at the time of harvesting (Mitchell & Moyle, 1951), and the water lying between them must therefore be equivalent to a layer only *c.* 20 mμ. thick. This water may simply be trapped between the cells, the viscous resistance preventing its escape during the one-hour period of centrifuging. However, other electrical or mechanical forces may also resist the closer approach of the osmotic barriers of the cells. There may, for instance, be a porous layer situated externally to the osmotic barrier. At all events, the behaviour of the suspensions of *M. pyogenes* in these experiments is in accord with the view that an osmotic barrier for inorganic phosphate exists near the external surface of the cells, and that this barrier envelops an internal space of $(2{\cdot}42 - 0{\cdot}76) \pm 0{\cdot}07 = 1{\cdot}66 \pm 0{\cdot}07$ ml./g., within which the acid-soluble inorganic phosphate is confined.

Phosphate exchange

Application of equation (7). In the system represented by equation (7), P_E and P_I are the total amounts of inorganic phosphate between which exchange occurs. These amounts, and also the total ^{32}P content of the system are considered to be constant. In other words, equation (7) is only applicable to the exchange experiments as long as a negligibly small proportion of the ^{32}P enters the organic fractions of the cells, and P_E and P_I remain constant. It was previously found (Mitchell & Moyle, 1953), that under highly aerobic conditions, *c.* 90% of the ^{32}P which entered resting cells at 25° in 90 min. was present in the acid-soluble inorganic fraction. About 1% of the ^{32}P entered the lipid and 10% entered the acid-soluble organic fraction. The present experiments were carried out under comparatively anaerobic conditions in narrow test tubes, with the aim of minimizing the residual metabolism and the entry of ^{32}P into the organic fractions.

The percentages of the total ^{32}P entering the inorganic and organic acid-soluble fractions, measured during phosphate exchange at 25° at pH 7 are shown in Table 3, together with the values of P_E and P_I. The acid-soluble organic faction was contaminated by a maximum of 3% of the inorganic fraction, and upper and lower figures for the ^{32}P content of the organic fraction are accordingly given in Table 3. There was very little change in the values of P_E and P_I, and even after 2 hr. 96% of the ^{32}P was still present in the inorganic acid-soluble fraction. The experimental values were therefore considered properly to represent P_E, P_I, $^{32}P_E$ and $^{32}P_I$ in the system described by equation 7.

Table 3. *The values of* P_E *and* P_I *and entry of* ^{32}P *into the organic fractions during phosphate exchange at 25° at* pH 7

Time (min.)	Percentages of total ^{32}P in the acid-soluble fractions		P_E (μmol./g.)	P_I (μmol./g.)
	Inorganic	Organic		
25	98	1·2–3·0	105	142
45	97	2·3–3·7	104	144
90	98	3·1–4·3	104	142
150	96	3·7–4·8	107	145

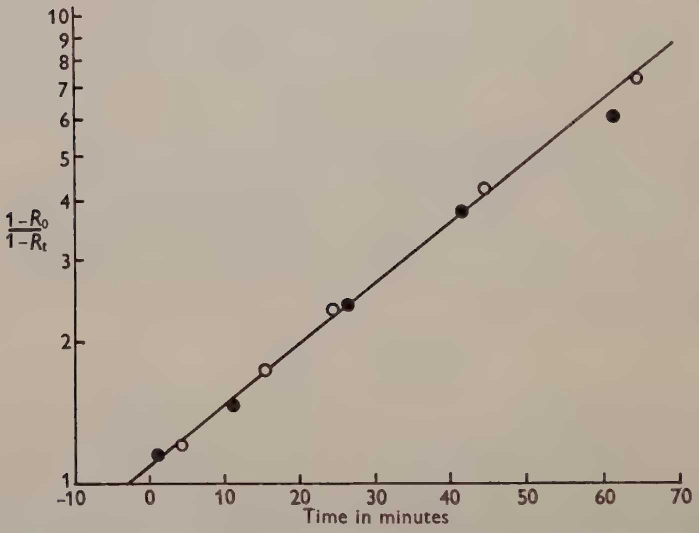


Fig. 2. Verification of equation (7). The plot of log $(1-R_0)/(1-R_t)$ against t; the ^{32}P moving into the cells, –●—●–; the ^{32}P moving out of the cells, –○—○–. At pH 7 and 25°.

It was now necessary to verify the relationship between $\dot{P}$, t and R described by equation (7). The time course of the changes in R during phosphate exchange at 25° at pH 7, with an external phosphate concentration of 2 mM, was observed with the ^{32}P moving from the medium to the cells and in the reverse direction. Fig. 2 shows the plots of log $(1-R_0)/(1-R_t)$ against t, the time being measured from the moment of mixing the washed suspension with the medium to the moment of starting the centrifuge. The points in this and in five other similar experiments (two of them at 15°) fell on straight lines, the slope of which was independent of the direction of movement of the ^{32}P, within experimental error. The intercept of the lines on the time axis (−3 min.) represents the effective time for which exchange continued during centrifuging. It was evident that the course of the phosphate exchange was described by equation (7), and that values of $\dot{P}$ could be estimated within a few per cent from a single determination of R after a known lapse of time $t+3$ min. The values of $\dot{P}$ at 25° at pH 7 varied between 3 and 6 μmole/g./min. in different batches of cells. However, in a given batch of washed suspension, measurements of $\dot{P}$ were reproducible to within ±5%, allowing a value of 15 min. for t.

The pH *value, temperature and ionic composition for* $\dot{P}$ *measurements.* The dependence of $\dot{P}$ on pH, determined under the conditions described above, is shown in Fig. 3. The pH values plotted are the means of the values measured in duplicate suspensions at the beginning and end of the period of phosphate exchange. It was noted that a downward drift of pH occurred at the rate of *c.* 0·1 unit/hr.

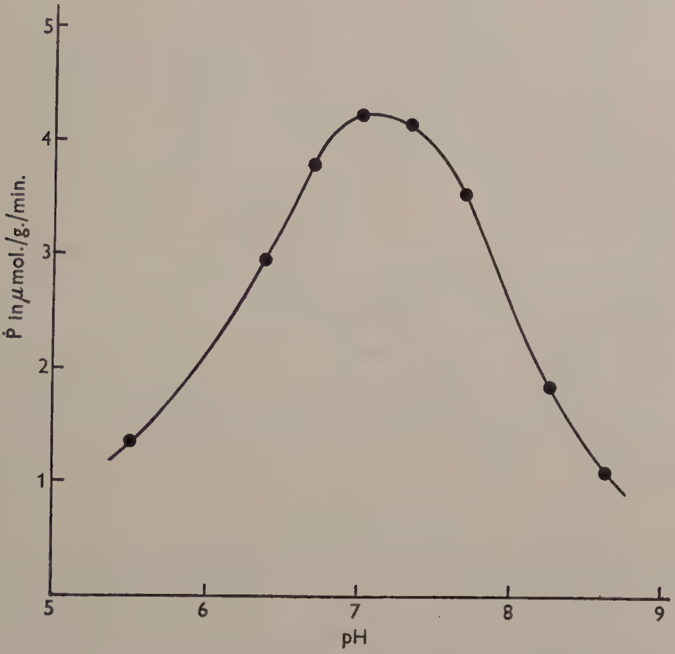


Fig. 3. The dependence of the rate of exchange of phosphate ($\dot{P}$) on pH in 0·1 M-NaCl and 2 mM-phosphate at 25°.

In order to make it easier to handle several tubes of suspension at a time in the inhibition studies, and to reduce errors of timing, exchange measurements were made at 15°. The value of $\dot{P}$ varied between 0·6 and 1·2 μmole/g./min. in different batches of cells, but in a given batch the measurements were reproducible to within ±5% allowing a value of 60 min. for t.

In previous work (Mitchell & Moyle, 1953) K^+ and Mg^{++} were present in the medium during phosphate exchange. It was found, however, that the addition of either 10 mM-KCl or 3 mM-$MgCl_2$, or of both simultaneously, to the saline used in the present experiments did not affect $\dot{P}$ significantly.

Inhibition of phosphate exchange. All the inhibition measurements were carried out at pH 7 at 15° in the presence of 0·1 M-NaCl and 2 mM-phosphate. About 100 'inhibitors' were tested, the majority of which had no effect or were effective only at relatively high concentrations. The less effective inhibitors provided information about the nature of the exchange system which it will be appropriate to consider in a later paper. Only the more potent group, the heavy metal cations, were of value in deciding between the exchange-adsorption and exchange-diffusion hypotheses. The percentage depression of $\dot{P}$ by cations added as chlorides to give an initial concentration of 10^{-4} M in a cell suspension of 20·3 mg./ml. was as shown in the following brackets: Phenyl-Hg^+ (72), Hg^{++} (48), Ag^+ (38), Cu^{++} (32), Cd^{++} (21), Co^{++} (10), Zn^{++} (32?). The ions Ca^{++}, Sr^{++}, Ba^{++}, Be^{++}, Mg^{++}, Mn^{++}, and Pb^{++} gave no depression. It was possible that, for some of the metals, an apparent effect on $\dot{P}$ might have been caused by precipitation of the sparingly soluble phosphate from the medium. The routine measurements showed, however, that the values of P_E were not affected in these experiments. Zinc, unlike any of the other metals, caused complete flocculation of the cell suspensions.

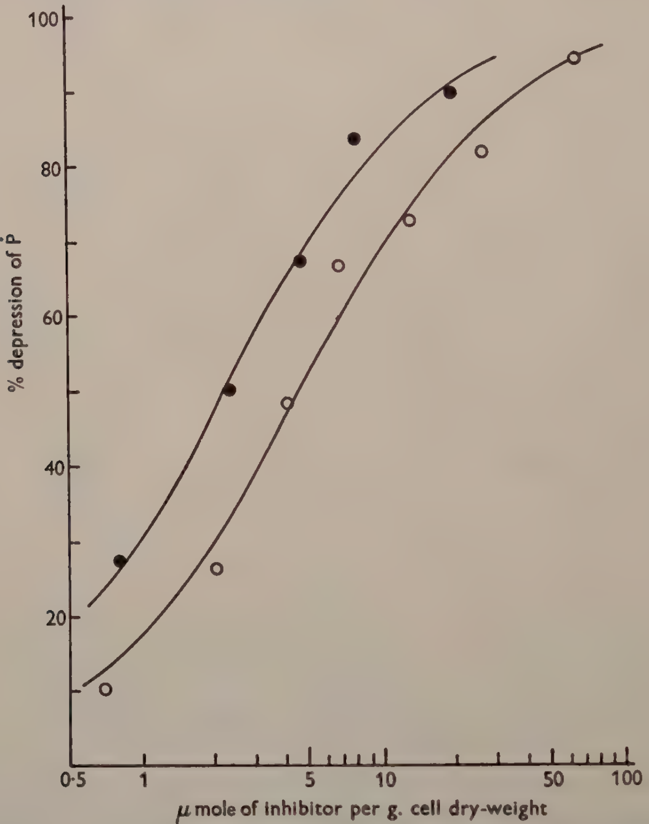


Fig. 4. The dependence of the rate of exchange of phosphate ($\dot{P}$) on the number of μmole of inhibitor present/g. cell dry-wt. Cell suspension density 18·7 mg./ml.; external phosphate concentration 2 mM; at pH 7 and 15°. For phenyl-HgCl (●) and $HgCl_2$ (○). Smooth curves given by $K = M.n/(100-n)$, M being amount of metal and n the percentage depression of $\dot{P}$; the constant K being 4·7 μmole/g. for $HgCl_2$ and 2·2 μmole/g. for phenyl-HgCl.

Although the zinc-containing suspension was redispersed every few minutes during phosphate exchange, it is probable that the loss of contact between the cells and medium gave an artificially large depression of $\dot{P}$. The dependence of the degree of depression of $\dot{P}$ on the amount of the two most potent inhibitors is shown in the semi-logarithmic plot of Fig. 4. The smooth curves represent the relationship $K = M.n/(100-n)$, M being the amount of metal ion, n the percentage depression of $\dot{P}$, and K a constant. The fact that the points lie close to the smooth curves shows that inhibition is the result of a reaction of the type $M+X \rightarrow MX$, X representing the sites which, when combined with metal (as MX), cause inactivation of a corresponding proportion of the units controlling the exchange of phosphate. In the experiments of Fig. 4, the cells contained 147 μmole acid-soluble inorganic phosphate/g., and the values of K were 4·7 μmole/g. for Hg^{++} and 2·2 μmole/g. for phenyl-Hg^{+}. Hence, the values of X per 100 molecules of acid-soluble inorganic phosphate must be 6·4 for Hg^{++} and only 3·0 for phenyl-Hg^{+}.

Evidently, therefore, the number of units controlling the exchange of phosphate is small in comparison to the number of adsorption sites of the exchange-diffusion hypothesis; and unless action at a distance were postulated, the observed inhibition could not be explained on the basis of exchange-adsorption. On the other hand, the results can be explained on the basis of an exchange-diffusion system in which the number of carrier groups which transfer phosphate across the osmotic barrier is not greater than 3 % of the total number of inorganic phosphate molecules present in the internal medium of the cells.

DISCUSSION

The evidence of the permeability and inhibitor studies is strong enough to warrant rejection of the exchange-adsorption hypothesis as it stands. Moreover, the behaviour of the system controlling the exchange of inorganic phosphate across the cell surface is entirely in accord with the exchange-diffusion hypothesis. It is important to realize, however, that we have not investigated what proportion of the inorganic phosphate of the internal medium of the cells exists as free ions. It is implicit in the exchange-diffusion hypothesis that the internal inorganic phosphate shall be free to react with the carrier groups in the osmotic barrier; but this does not necessarily imply that the internal inorganic phosphate must exist as free ions. Under the conditions of the present experiments, 1 g. dry-weight of cells contained *c.* 150 μmole inorganic phosphate in an internal space of 1·66 ± 0·07 ml. Hence, the phosphate concentration in the internal medium was *c.* 0·1 M. If this were to exist as the completely dissociated alkali salts at pH 7, the osmotic pressure would be slightly above 5 atmospheres—a considerable pressure for only one of the many solutes which constitute 18 % of the cell dry-weight. As a whole, it will be seen that the internal solutes form about a 10 % (w/v) solution. Discussion of the osmotic pressure exerted by the internal solutes in relation to the stress on the cell envelope should await osmotic measurements which are now in progress in this laboratory. It would be appropriate to remark here, however, that for a sphere the size of *M. pyogenes*, 0·7 μ. in diameter, an internal pressure of 5 atmospheres corresponds to a stress of 87 dynes/cm., only slightly above the surface tension of water. The cell envelope, being made of a protein similar in composition to silk fibroin (Mitchell & Moyle, 1951), may be able to withstand tensions much greater than this.

We might reasonably expect that a considerable proportion of the inorganic phosphate of the internal medium of the cell would occur in association with polyvalent cationic components; but there is at present no justification for assuming that the bulk of it is specifically bound.

It has been stated by Roberts & Roberts (1950), that the exchange of inorganic phosphate across the surface of *Bact. coli* is controlled by exchange-adsorption. We might therefore conclude that the mode of uptake and the state of the 'inorganic phosphate' in *Bact. coli* differs fundamentally from that in *M. pyogenes*. The main objection to the exchange-diffusion hypothesis is the statement of Roberts & Roberts (1950) and of Roberts & Wolfe (1952) that *Bact. coli* is freely permeable to inorganic phosphate. So far, however, this statement has not been supported by experimental evidence amenable to direct comparison with the observations described in this paper. Under the circumstances, it would be advisable to await a parallel examination of the phosphate exchange systems in *Bact. coli* and *M. pyogenes* by the same group of workers before concluding that they differ fundamentally.

The occurrence of the reciprocal exchange of phosphate across the osmotic barrier of *M. pyogenes* under resting conditions may offer a special opportunity for studying the mechanism whereby inorganic phosphate is passed inward through the osmotic barrier against a concentration gradient when a source of free energy is available. A closer examination of the exchange-diffusion system in resting *M. pyogenes* has therefore been undertaken.

REFERENCES

Conway, E. J. & Downey, M. (1950). An outer metabolic region of the yeast cell. *Biochem. J.* **47**, 347.

Green, D. E., Atchley, W. A., Nordman, J. & Tepley, L. J. (1949). Studies on the cyclophorase system. XII. Incorporation of ^{32}P. *Arch. Biochem.* **24**, 359.

Harman, J. W. (1950). Studies on mitochondria. II. The structure of mitochondria in relation to enzymic activity. *Exp. Cell Res.* **1**, 394.

Kamen, M. D. & Spiegelman, S. (1948). Studies on the phosphate metabolism of some unicellular organisms. *Cold Spr. Harb. Symp. quant. Biol.* **13**, 151.

Krebs, H. A., Eggleston, L. V. & Terner, C. (1951). *In vitro* measurements of the turnover rate of potassium in brain and retina. *Biochem. J.* **48**, 530.

Lundegårdh, H. (1945). Absorption, transport and exudation of inorganic ions by the roots. *Ark. Bot.* **32** A, 12, 1.

Mitchell, P. & Moyle, J. (1951). The glycerophospho-protein complex envelope of *Micrococcus pyogenes*. *J. gen. Microbiol.* **5**, 981.

Mitchell, P. & Moyle, M. J. (1953). Paths of phosphate transfer in *Micrococcus pyogenes*: phosphate turnover in nucleic acids and other fractions. *J. gen. Microbiol.* **9**, 257.

Roberts, R. B. & Roberts, I. Z. (1950). Potassium metabolism in *Escherichia coli.* III. Interrelationship of potassium and phosphorus metabolism. *J. cell. comp. Physiol.* **36**, 15.

Roberts, R. B. & Wolffe, E. L. (1952). Utilization of labelled fructose-6-phosphate and fructose-1,6-diphosphate by *Escherichia coli. Arch. Biochem.* **33**, 165.

Ussing, H. H. (1947). Interpretation of the exchange of radio-sodium in isolated muscle. *Nature, Lond.* **160**, 262.

(*Received* 28 *March* 1953)

FARRANT, J. L. & ROUNTREE, P. M. (1953). *J. gen. Microbiol.* 9, 288–292.

Electron Microscopy of a Staphylococcal Bacteriophage

BY J. L. FARRANT

Chemical Physics Section, Division of Industrial Chemistry, Commonwealth Scientific and Industrial Research Organization, Melbourne

AND PHYLLIS M. ROUNTREE

Fairfax Institute of Pathology, Royal Prince Alfred Hospital, Sydney

SUMMARY: The staphylococcal bacteriophage 3A consists of a flat, oblong head, *c.* 600A. by *c.* 1000A., and a long tail, *c.* 120A. by *c.* 2900A. Fixation with formalin destroys the integrity of the head. Cells infected with the phage release it at the end of the latent period by a process resembling the bursting of a bag. After the phage is released, the empty cell membranes persist for a short time before being dissolved in the medium. Correlation of these findings with turbidity readings and Gram staining of the infected cultures indicate that the turbidity falls only when the shells of the cocci are dissolving.

Electron microscopy has been used extensively to study the morphology of the coli T series of bacteriophages and the processes of phage adsorption and cellular lysis with these particular host-virus systems. There have been fewer studies of staphylococcal bacteriophages. Wyckoff (1949) published one photograph of the staphylococcus phage K. Included in the paper of Smiles, Welch & Elford (1948) were unshadowed photographs of cells lysed by phage K in which the phage particles are arranged radially around the remnants of the cells. Hotchin, Dawson & Elford (1952) photographed phage K adsorbed tail first to the cell membranes of staphylococci that had been previously disrupted ultrasonically. This paper is concerned with the staphylococcal phage 3A, originally isolated by Wilson & Atkinson (1945), the growth characteristics of which have been described elsewhere (Rountree, 1952). The object of the investigation was to study the morphology of the phage and the release of the phage from infected cells.

EXPERIMENTAL

Preparation of the material

The material used consisted of:

(1) Phage adsorbed to the surface of heat-killed cells, washed in 0·002 M-$CaCl_2$ and twice in distilled water before being placed on the collodion supporting films.

(2) Staphylococci from broth cultures to which sufficient phage had been added to produce infection of all cells. Samples were removed at the end of the one-step growth curve when titrations showed that extracellular phage was increasing. The samples were centrifuged and the cells then fixed in 0·2 % (v/v) formalin or in 0·5 % (w/v) osmium tetroxide, and washed twice in water before mounting.

Unshadowed phage could not be observed satisfactorily, so the preparations were shadowed with uranium at an angle $\tan^{-1} \frac{1}{4}$. The specimens were examined with an RCA type EMU electron microscope which had been adjusted to minimize all instrumental factors known to detract from the resolution.

RESULTS

Morphology of the phage and its adsorption

Pl. 1, fig. 1, shows phage 3A adsorbed to the surface of heat-killed staphylococci. Some unadsorbed particles are present in the field. The particles are characterized by the possession of a flat oblong head, the average dimensions of which are *c.* 600 A. wide and *c.* 1000 A. long, and an apparently flexible tail *c.* 120 A. wide and *c.* 2900 A. long. The heads contain little electron-scattering material and for this reason are difficult to observe in unshadowed preparations. The collapsed appearance of many of the heads suggests the presence of a limiting membrane around them. The majority of the particles are adsorbed to the surface of the cocci tail first. There is also evidence of the disruption of the coccal cell wall. In this preparation an average of 60–70 particles were adsorbed to each coccus. In preparations with an average of 10 particles per coccus, disruption of the cell wall was not obvious.

Release of phage from infected cells

Pl. 1, fig. 2, shows cocci fixed in the act of releasing phage. All such cocci that we have observed appear to lyse in one place only, as if the cell wall were torn thus producing a gap through which poured out a stream of phage particles mixed with smaller macro-molecular particles. The smaller particles are similar in size and appearance to those obtained by Hotchin *et al.* (1952) from ultrasonically disrupted uninfected cells and are probably normal constituents of the cell cytoplasm. In Pl. 1, fig. 2, there is also an intact coccus; the differences between the diameters of the two cocci and the lengths of the shadows cast by them indicate that the release of phage was accompanied by flattening of the coccus. After the phage had been released the walls of the cocci remained for a time as empty shells, a characteristic example of which is shown in Pl. 2, fig. 3. Sometimes one member of a pair of cocci was found intact with the remnants of the other coccus attached to it.

The effect of fixation with formalin

In Pl. 1, figs. 1 and 2, the material was either unfixed or fixed with osmic acid. In obtaining Pl. 2, fig. 3, formalin was used as the fixative. In such preparations marked alterations in the heads of the phage particles were apparent. The particles appeared mostly either as tails lacking heads Pl. 2, fig. 3, or else the heads, when present, were flattened and hazy in outline Pl. 2, fig. 4. It has been known for many years (Burnet, Keogh & Lush, 1937) that formalin destroys the infectivity of phage without affecting its antigenicity. The present photographs indicate that the action of the formalin is to rupture the limiting membrane of the phage heads which then disintegrate.

Correlation of electron microscope appearances with those observed with the optical microscope

One of the characteristics of phage 3A is that broth culture in which every coccus has been infected increases in turbidity during the latent period of 50 min.; then follows a period of 15–25 min. during which the turbidity remains stationary or falls only slightly, but during which the infective phage is released from the cocci. Rapid clearing of the cultures takes place only after the phage has been liberated. In certain cases where a low yield of phage/coccus is obtained the clearing may be prolonged beyond 3 hr. Phage 3A therefore resembles the *Staphylococcus muscae* phage used by Price (1948, 1949, 1952), and the *Bacillus megaterium* phage of Northrop (1952) when grown in certain media.

When samples of phage 3A-infected cultures were removed 75 min. after infection, stained by Gram's method and examined with the light microscope, the appearances observed could be correlated with those seen with the electron microscope. The smears contained large numbers of Gram-positive cocci, some of which were embedded in a matrix of Gram-negative material. The majority of these cocci on careful examination revealed distorted outlines and in a few cases a smear of Gram-negative material was seen apparently emerging from one side of the cell. These distorted cells seem to correspond to the bursting cells of Pl. 1, fig. 2. Also present in the smears were Gram-negative sac-like objects which were sometimes attached to a Gram-positive coccus. Occasionally these sacs had around their edges crescentic areas that were Gram-positive. The sacs appear to correspond to the empty shells of the cocci shown in Pl. 2, figs. 3 and 4. Smears taken later, when clearing of turbidity was proceeding rapidly, showed large amounts of Gram-negative debris and a very few distorted cocci. When clearing was complete, nothing could be seen in the smears.

DISCUSSION

It has been claimed by Price for the *Staph. muscae* phage, and by Northrop for a *B. megaterium* phage that these phages may be liberated from infected cells without cellular lysis, although such cells do ultimately lyse. The criteria of cellular lysis used by Price were turbidity readings and microscopic examination of smears stained with methylene blue; Northrop's criterion was turbidimetric. Lwoff & Gutmann (1950) suggested that Price's results might have been due to lysis of the cocci during the dilution process necessary before phage titration. This suggestion was disposed of by the demonstration of free phage in the supernatants of the infected cultures. Without examination by electron microscopy, claims similar to those of Price and Northrop could have been made for phage 3A. However, phage 3A is in fact released by the bursting of the infected coccus and the ejection of its contents into the surrounding medium. The shells that remain after the phage is released stain Gram-positive for a short time, gradually lose their Gram positivity and eventually disappear. The turbidity of the cultures, as measured by a photoelectric colorimeter, remains stationary or falls only slightly during the period of phage release and falls rapidly only when the shells of the cocci are dissolving. In these cultures, therefore, turbidity is not an adequate criterion of the integrity of the infected cocci.

It would seem that some confusion has been caused by using synonymously the terms 'cellular lysis' and 'phage release'. In most bacteriophage systems these events occur simultaneously and the dramatic disappearance of the bursting cells can be observed with the optical microscope. It has therefore been natural to regard the two events as closely correlated. But with some phages, and in certain media, release of phage may precede complete dissolution of the cells, so that the two events are separated in time. In such cases, however, there is no need to postulate a mechanism of phage release other than the normal bursting of infected cells. In any case, the term 'cellular lysis' is frequently employed to describe types of lysis which occur under conditions other than bacteriophage infection. It might be preferable to use the term 'cellular bursting' for the process of phage release.

It is perhaps significant that the phage systems in which this delayed dissolution of infected cells occurs are those in which the host cells are Gram-positive. Gram-positive cells may possess a cell wall more rigid than that of the Gram-negative organisms in which phage release occurs at the same time as dissolution of the cells. The phenomenon is, however, complex, since different types of media may influence the time of lysis, as may also the use of different but closely related host cells.

Some investigations have suggested (see Bartholomew & Mittwer, 1952) that the property of Gram-positivity may reside in the cell wall of Gram-positive bacteria, but there is some doubt as to whether the cytoplasm of these cells may or may not be Gram-positive. The Gram-negative character of the material extruded from the 3A-infected cocci, the walls of which were still Gram-positive, suggests that the cytoplasm of these cocci may be Gram-negative. However, the results should be interpreted with caution since the internal organization of these cocci may have been profoundly altered by the process of phage multiplication.

This work was assisted by a grant to one of us (P. M. R.) from the Australian National Health and Medical Research Council.

REFERENCES

Bartholomew, J. W. & Mittwer, T. (1952). The Gram stain. *Bact. Rev.* **16**, 1.

Burnet, F. M., Keogh, E. V. & Lush, D. (1937). The immunological reactions of the filtrable viruses. *Aust. J. exp. Biol. med. Sci.* **15**, 231.

Hotchin, J. E., Dawson, I. M. & Elford, W. J. (1952). The use of empty bacterial membranes in the study of the adsorption of Staphylococcus K phage upon its host. *Brit. J. exp. Path.* **33**, 177.

Lwoff, A. & Gutmann, A. (1950). Recherches sur un *Bacillus megaterium* lysogène. *Ann. Inst. Pasteur*, **78**, 711.

Northrop, J. H. (1952). The effect of various culture media on infection, growth, lysis and phage production of *B. megaterium*. *J. gen. Physiol.* **35**, 471.

Price, W. H. (1948). Phage formation in *Staphylococcus muscae* cultures. II. The release of the virus from the bacterial cell. *J. gen. Physiol.* **32**, 203.

Price, W. H. (1949). Phage formation in *Staphylococcus muscae* cultures. V. Further observations on the relationship between virus release and cellular lysis. *J. gen. Physiol.* **32**, 481.

Price, W. H. (1952). Phage formation in *Staphylococcus muscae* cultures. X. The relationship between virus synthesis, the release of bacterial ribonucleic acid, virus liberation and cellular lysis. *J. gen. Physiol.* **35**, 409.

Rountree, P. M. (1952). Serological studies of the multiplication of a staphylococcal bacteriophage. *Aust. J. exp. Biol. med. Sci.* **30**, 567.

Smiles, J., Welch, F. V. & Elford, W. J. (1948). The influence of antibacterial substances on the interaction of bacteria and bacteriophages. 2. Optical studies of the penicillin effect. *J. gen. Microbiol.* **2**, 220.

Wilson, G. S. & Atkinson, J. D. (1945). Typing of staphylococci by the bacteriophage method. *Lancet*, i, 647.

Wyckoff, R. W. (1949). *Electron microscopy.* New York: Interscience Publishers.

EXPLANATION OF PLATES

Plate 1

Fig. 1. Staphylococcal phage 3A particles adsorbed on to heat-killed cocci of *Staph. aureus*. Not fixed. × 30,700.

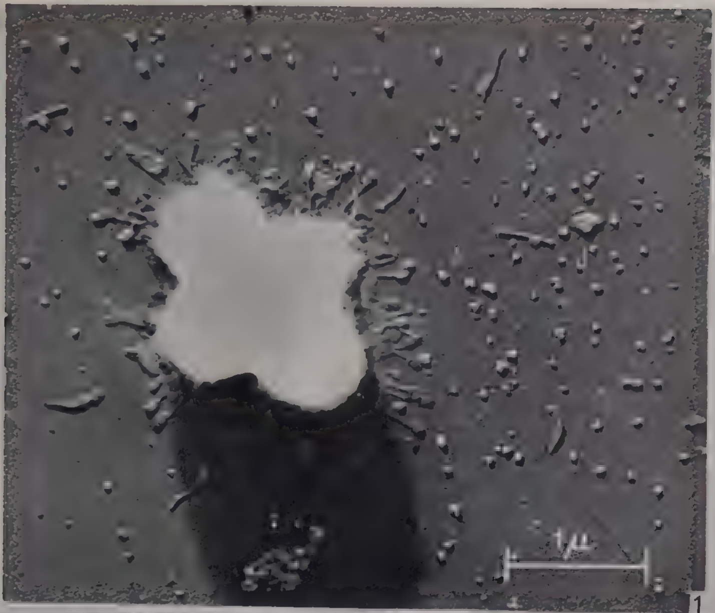
Fig. 2. A burst and an intact cell of *Staph. aureus*. Fixed with osmium tetroxide. × 29,600.

Plate 2

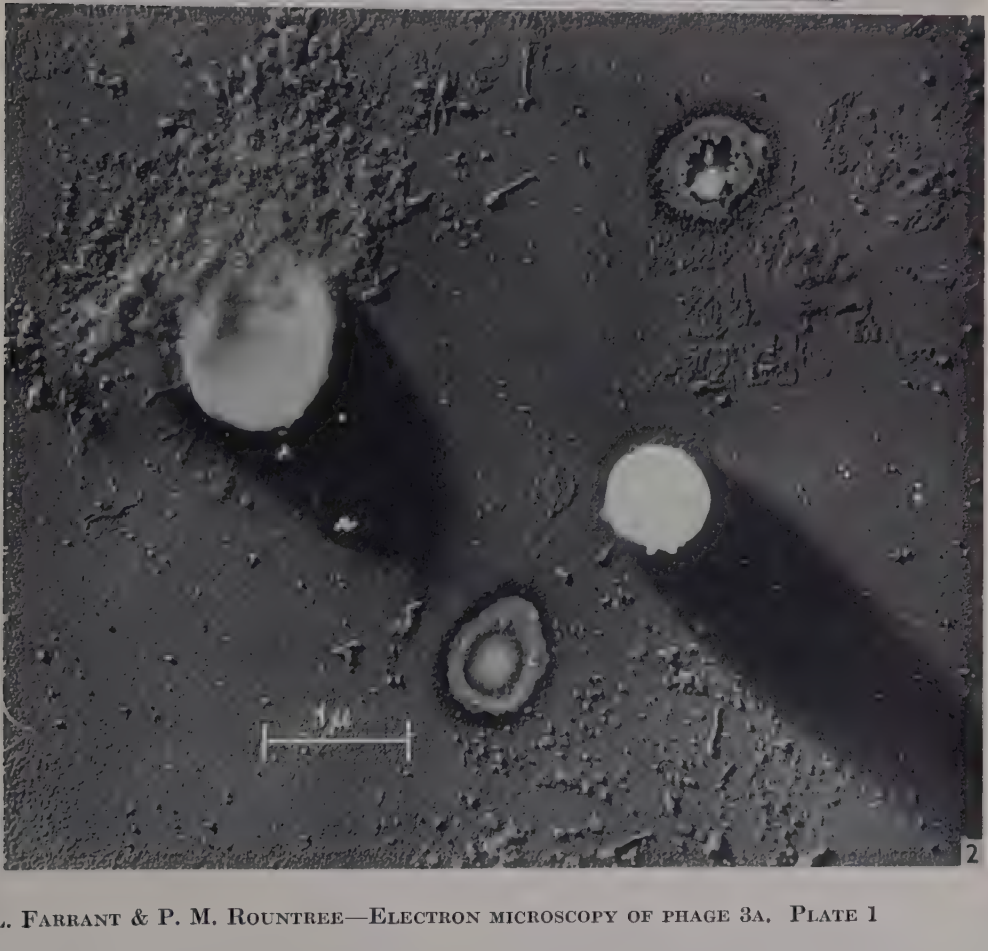
Fig. 3. An almost empty shell from a burst coccus. Most of the phage particles present lack heads. Fixed with formalin. × 30,000.

Fig. 4. Disintegrating shells from burst cocci. The heads of the phage particles in this field are flattened. Fixed with formalin. × 29,400.

(*Received* 1 *April* 1953)

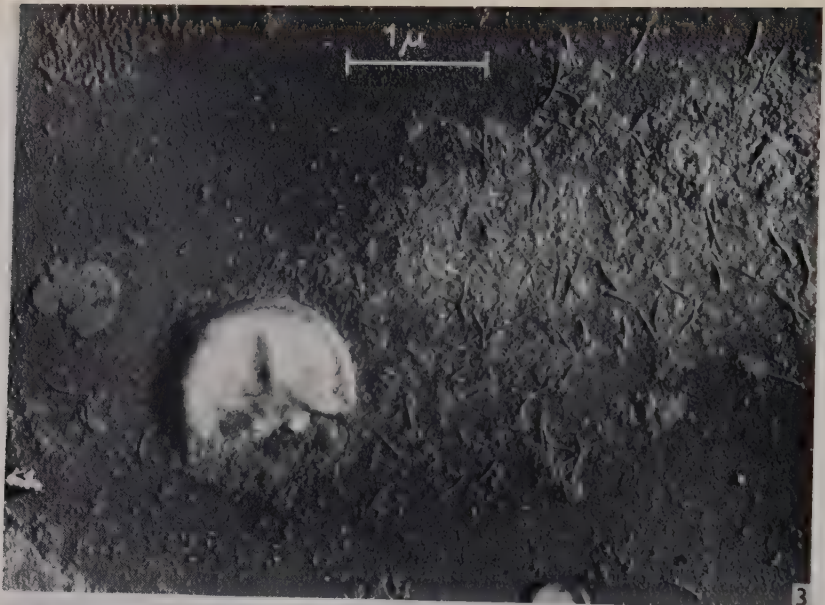


1

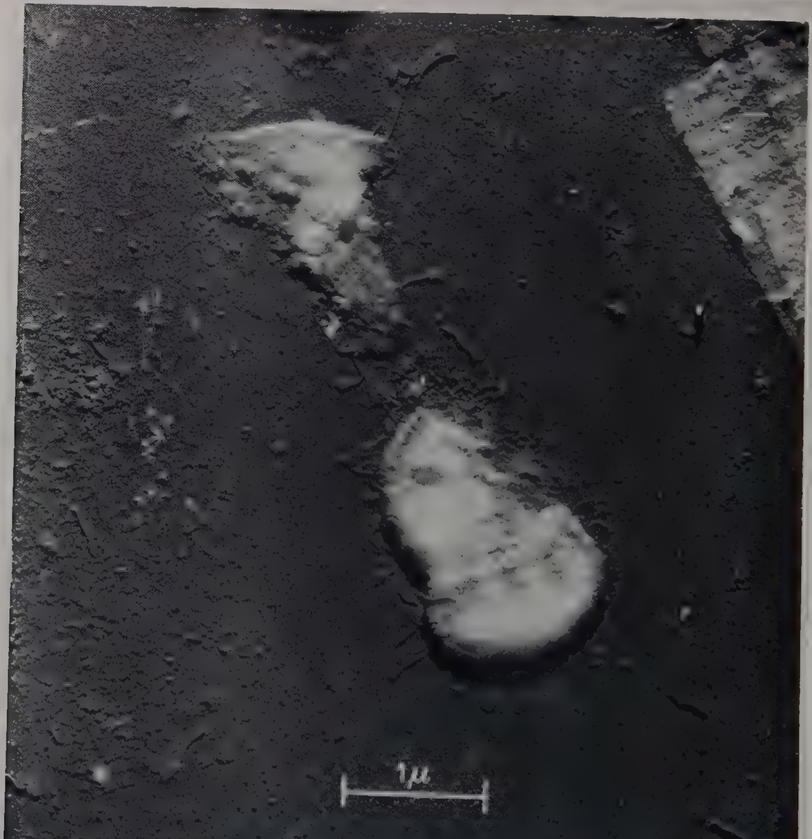


2

J. L. Farrant & P. M. Rountree—Electron microscopy of phage 3a. Plate 1



3



4

J. L. Farrant & P. M. Rountree—Electron microscopy of phage 3A.
Plate 2

Johnstone, K. I. (1953). *J. gen. Microbiol.* 9, 293–304.

Micromanipulation on an Agar Surface for the Isolation and Cultivation of Single Organisms

By K. I. JOHNSTONE

Department of Bacteriology, University of Leeds

SUMMARY: Organisms may readily be isolated and cultured separately by micromanipulation on the upper surface of an agar block. A micromanipulator is not essential, its place being taken by a second microscope with simple attachments. Illumination methods by dark-ground and phase-contrast techniques are described. A modification using a simple micromanipulator enables isolations to be made very rapidly. A device is described for making the necessary micro-instruments in a standard microscope.

The cultivation of single organisms or their spores by manipulation on the upper surface of an agar block has several advantages: (1) the organisms show clearly by dark-ground or phase-contrast illumination; (2) the microneedles, being above the agar surface, are constantly visible, centration is easy and accidents are minimized; (3) the selected organism is carried 0·5–1·0 cm. across a sterile agar surface, giving many opportunities to observe that it is single, and contamination from the other organisms is eliminated; (4) the position of the isolated organism is marked by melting pits in the agar around it (Johnstone, 1943), which, being visible to the unaided eye, enable the agar block to be dissected and the single organism to be carried on a portion of the block to any suitable medium for culture; (5) a micromanipulator is not essential but it can be used to accelerate the isolations. The disadvantage is that dry lenses only can be used, with a maximum useful magnification of × 600, which is, however, adequate for most bacteria. The method has been successfully used for bacteria and their spores, the spores of fungi, amoebae and their cysts.

APPARATUS

The agar block. Either nutrient agar or a plain agar gel can be used, but it must be very clear, especially for dark-ground illumination. A 3 × 1 in. slide is mounted above a 3 × 1·5 in. slide, being separated from it at each end by a glass strip 2 mm. thick, the whole being clamped by wire paper clips and sterilized by dry heat in a Petri dish. After warming the slides to 60°, melted agar is run into the space between them with a bent capillary pipette and is allowed to set. The clips are removed, the upper slide is slid off and the agar slab is cut into blocks 2·5 × 1·0 cm. with a sterile stainless steel knife. Each block is lifted with the knife on to a sterile 3 × 1·5 in. slide enclosed in a Petri dish, with moist cotton wool wads to prevent drying and distortion of the plane agar surface.

A sterile Perspex cell (Fig. 1) enclosing an area 1·6 × 3·5 cm. protects the agar from dust and decreases evaporation. For methods 1 and 2 an aperture *A* at one end admits the microneedle shaft, and the cover is of Perspex with a central raised collar *B* which just admits the nose of the 4 mm. objective. The cover slides in grooves in the cell, thus allowing movement of the objective relative to the cell and agar during manipulation in the lateral direction, whilst the whole cell moves on the slide during the short transverse movements. For method 3, the cover is replaced by a sterile rectangular cover-glass 1 × 2 in. and a second opening is provided for the marking needle, at the right-hand end of the cell, when a micromanipulator is used.

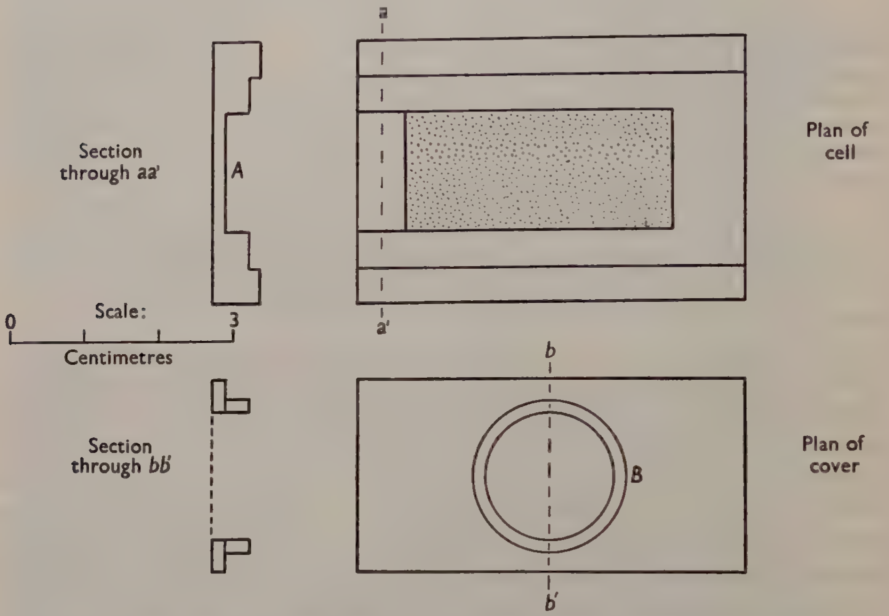


Fig. 1. The Perspex cell and cover. The enclosed area of the slide is stippled. *A*, aperture for microneedle shaft; *B*, collar admitting objective.

The manipulating microscope. This replaces a conventional micromanipulator and must have sensitive coarse and fine adjustments. The following attachments are used:

(1) *The nosepiece attachment.* A brass plug *A* (Fig. 2), with the R.M.S. objective thread, is screwed into the rotating nosepiece. The plug carries a brass arm *B*, rotating about the screw *C* and locked by the clamp nut *D*, so that the arm lies across the microscope stage with the socket *E* on the right-hand side when the plug is in the optical axis. A screw *F* clamps the microneedle-holder, or the marking needle, in the socket *E*.

(2) *The microneedle-holder.* A steel rod *G* (Fig. 2) fits into the socket *E* of the nosepiece attachment. The terminal portion *H*, 3 cm. long, is reduced to 2·5 mm. diam. and carries a socket with clamp screw *I* for the handle of the glass microneedle. A knurled brass collar *J* enables the holder to be rotated for correct orientation of the needle tip. The length from the optical axis to *I* should be approximately 14 cm.

(3) *The microneedle.* The most useful type for the isolation of bacteria, bacterial spores and organisms of similar size has (*a*) a handle 2 cm. long and 1 mm. in diameter, drawn from 6 mm. soft glass rod by hand; (*b*) a shaft 2·5 cm. long and 0·1–0·2 mm. in diameter, drawn out over a microburner and inclined slightly downwards relative to the handle so as to clear the agar surface except at the tip; (*c*) a tip, inclined to the agar surface at an angle of 30° and 1–5 μ. in diameter at the end. (The microburner is simply a minute coal-gas flame burning at the end of a vertical fine capillary tube.) Simple tips can be made merely by touching the end of the shaft momentarily against an electrically heated platinum filament (36 s.w.g.) at bright red heat and by drawing away at an angle. They are best made, however, under microscopic observation as described below.

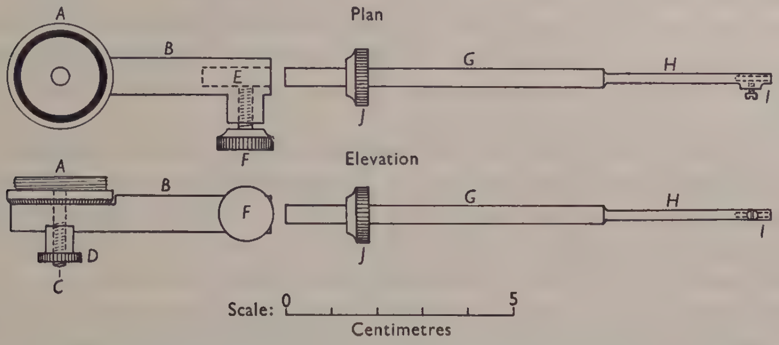


Fig. 2. The nosepiece attachment (left) and microneedle-holder (right).

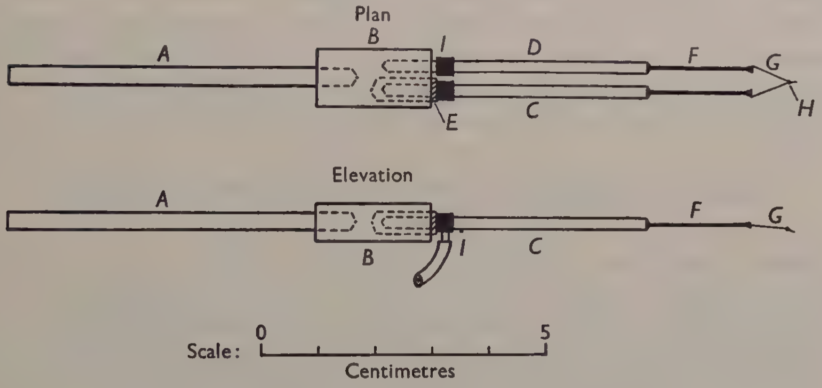


Fig. 3. The platinum marking needle and mount.

(4) *The marking needle.* The steel rod *A* (Fig. 3) fits into the socket of the nosepiece attachment and carries a brass block *B*, with two steel rods *C* and *D*, 2·5 mm. in diameter, one of which (*C*) is electrically insulated by a plastic or ebonite sleeve *E*. Into the ends of *C* and *D* are soldered thick platinum wires *F* (24 s.w.g.) 2 cm. long, which carry the heating element *G* made from two fine platinum wires (36 s.w.g.), one end of each being welded into the

hooked and flattened end of the corresponding thick wire by tapping the joint with a steel rod whilst at white heat in the blowpipe flame. The free end of one wire is hooked around the other at *H* and welded to it, the terminal 2 mm. of the second wire being bent down at an angle of 45° to form the needle tip. The sides of the heating element should be 7 mm. long. Connexion is made to the steel rods at *I* with fine silk-covered wire from a 3 V. transformer (*a*) directly, to raise the 'V' of the element to bright red heat, thus sterilizing the tip by conduction, and (*b*) through a resistance such that the tip is just sufficiently warmed to melt the agar, thus forming the locating pits without causing spluttering of the agar.

Sterilization of the apparatus. The Perspex cell, cover-glass and microneedles are sterilized by exposure for 6 hr. to the vapour of commercial formalin in a desiccator, the upper section being warmed by a 6 V., 3 watt electric bulb to prevent condensation on to the articles. The apparatus is then stored in sterile Petri dishes overnight, when all formaldehyde vanishes.

The observing microscope. This must have a rigid mechanical stage, free from backlash, and preferably an inclined eyepiece since the stage must be horizontal. A 4 mm. objective is used with a maximum N.A. of 0·70 to give sufficient working distance for the microneedle to operate. This aperture is reduced to 0·60–0·65 for dark-ground illumination, or when a long-working-distance attachment is used. A 16 mm. objective is centred on the nosepiece relative to the 4 mm. lens and is mounted on an extension tube to make it par-focal with the long-working-distance attachment if this is used.

Method 1. *Dark-ground illumination with short working distance*

This is the simplest method, the organisms being well illuminated and appearing larger than by other methods. The disadvantages are (1) the excessive glare from the needle tip often obscures the organism; (2) the intense illumination may cause condensation on the objective due to local heating of the agar, but viability of the cells is usually unimpaired if ultraviolet light is filtered out. Two condenser systems are satisfactory: (*a*) the long-working-distance dark-ground condensers (e.g. the dry 'Zonal' of Watson); (*b*) an achromatic condenser can be adapted to give a long working distance of 3 mm. to focus through slide and agar block (Goldie, Gordon & Johnstone, 1948).

Method 2. *Phase-contrast illumination with short working distance and optional dark-ground illumination*

The advantages are (1) the microneedle appears without marked glare; (2) the intensity of illumination and heating of the agar are much reduced; (3) organisms can be selected which show marked positive phase-contrast and these (type *A* of Pulvertaft) are more likely to be viable (Pulvertaft, 1952). A 4 mm. phase-contrast objective with adequate working distance is used and a phase-contrast condenser with at least 3 mm. working distance. Alternatively an achromatic condenser modified to give this working distance can be used, provided that the lower focal plane is accessible for the insertion of an annulus to register with the phase-plate of the objective. If two concentric annuli are used, phase-contrast illumination is obtained by partially closing the iris diaphragm, thus obscuring the outer annulus; dark-ground illumination by obscuring the inner annulus with a patch stop and opening the iris. This transition is valuable during manipulation of organisms. A similar transition is obtained by different means with recent types of condenser by Leitz and Zeiss-Winkel. The inner annulus automatically gives dark-ground illumination with the 16 mm. lens.

Method 3. Phase-contrast illumination with long working distance

A 4 mm. phase-contrast objective is carefully centred within a reflecting attachment (Newton), which lengthens the working distance to 1 cm. The advantages are (1) all manipulations can be done under a sterile cover-glass, greatly decreasing air contamination; (2) the inclined tips of the microneedles can be lengthened, thus raising the shafts well above the agar, to which they may otherwise adhere by capillarity if lowered too far, thus spoiling the agar block; (3) with a micromanipulator the marking needle can be kept in position close to the microneedle throughout the isolations, thus saving much time in recentring the needles.

The disadvantage is the great loss of light in the mirror system, necessitating a powerful lamp (e.g. a 12 V., 60 watt bulb with concentrated filament) and an aplanatic lamp condenser. The beam should be cooled either by a thick water trough or a 3 mm. thickness of Chance's ON 20 glass, to avoid heating the agar. This is the method of choice.

TECHNIQUE

(1) *Inoculation of the agar block*

A faintly turbid suspension of the organisms is made in sterile distilled water. A sterile brass protecting screen 1 cm. high, carried on feet so that its lower edge just clears the agar surface, is placed across the agar block, one-quarter of the length of the latter from the right-hand end, with sterile forceps. This catches droplets of the suspension liberated as the water column between the loop and the agar breaks after inoculation, which may otherwise contaminate the adjacent sterile agar surface. A small (1 mm.) loopful of the suspension is drawn across the right-hand end of the block, without touching the plane agar surface with the loop. Contact is maintained by the column of water between loop and agar, and the surface is not deformed. The fluid evaporates or is absorbed and the screen is removed. Organisms from the left-hand margin of the inoculum are used for manipulation, being readily identified as a well-defined line (Fig. 4*a*). The sterile cell and cover are placed over the block.

(2) *Setting up of the agar block and centration of the microneedle*

The slide, with block and enclosing cell, is placed on the mechanical stage, the condenser is focused and the left margin of the inoculum is located with the 16 mm. objective. The 4 mm. objective is then lowered through the cell cover (or simply rotated into the axis for method 3) and the margin of the inoculum is centred in the field. The objective is then raised in methods 1 and 2 for insertion of the microneedle.

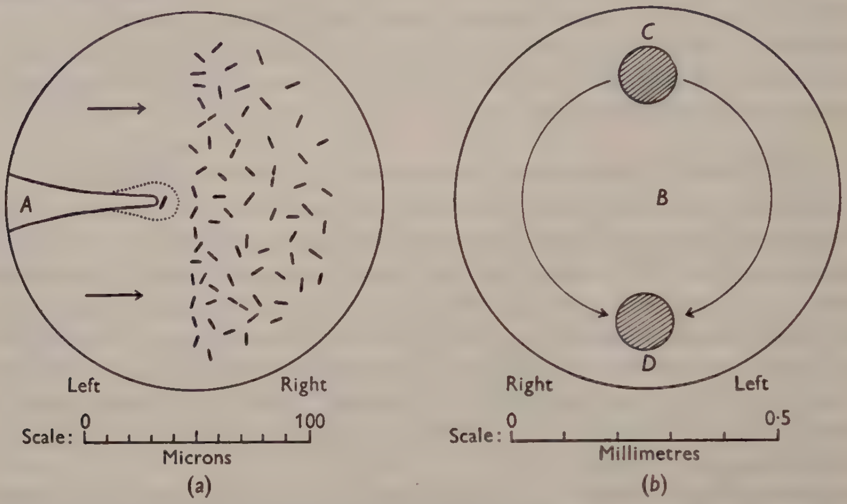


Fig. 4. (*a*) A single organism being drawn away from the edge of the inoculum. The cone of water around the needle tip *A* is shown by the broken line. Direction of movement of the agar is shown by the arrows. Method 3. No apparent image reversal: 4 mm. phase-contrast objective. (*b*) The positions of the locating pits *C* and *D* in the agar surface relative to the isolated organism *B*. The arrows show the alternative courses taken by the marking needle. The microneedle has been removed. Image reversed: 16 mm. objective.

The manipulating microscope is placed on the left of the observing microscope and, by sliding it on the smooth bench, the sterile microneedle carried in the needle-holder is introduced through the aperture in the cell. The tip is lowered by the coarse adjustment of the manipulating microscope until just above the agar surface and is centred in the field.

(3) *Manipulation of the organism*

The objective and microneedle are lowered alternately in stages, *first* the objective and *secondly* the microneedle, using the coarse adjustments, until the organisms come into focus. The needle is then lowered with the fine adjustment until it just touches the agar, when it draws a cone of water from the gel (Fig. 4*a*). With methods 1 and 2 the image is reversed, but with method 3, owing to the additional reversal in the mirror system, objects appear to be in their actual positions and manipulation is simplified.

The principle, based on that of Koblmüller & Vierthaler (1933), is that if the needle tip *A* (Fig. 4*a*) is lowered over an organism, it will float in the cone of water around the tip and can be carried for 1 cm. or more across the sterile agar by operating the mechanical stage, thus moving the agar relative to the needle as indicated by the arrows in Fig. 4*a*. The needle point remains centred in the field, its height being constantly regulated by the fine adjustment of the manipulating microscope. Should the organism escape from the cone, the needle is raised, the agar is moved back, the needle is lowered over the organism and progress continued. To assist recognition of the escape of the organism, the needle point should be set slightly to one side in the field. The organism is constantly presented in different positions and the presence of a second organism evident.

For filamentous organisms which will not follow the needle, alternative methods are used: (1) a hooked microneedle encircles the organism at the middle of its length and drags it along (Johnstone, Crofts & Evans, 1950); (2) the normal tip is used to lift the filament from the agar surface and to deposit it on a sterile area. It is then straightened and examined to ensure that no other organisms are present, is again lifted and carried to its final position where it is located in the usual way.

(4) *Marking the site of the isolated organism*

When at least 5 mm. from the inoculum, the position of the organism is marked by melting two or more pits in the agar surface around it. For methods 1 and 2, the microneedle is raised slightly and, with the 16 mm. objective, the organism is located by dark-ground illumination close to the tip of the needle. The microneedle is withdrawn from the cell and the needle-holder is replaced by the platinum marking needle, which is sterilized by the heating current applied for 2 sec. The needle tip is centred in the field, is pressed slightly into the agar surface to one side of the organism (Fig. 4*b*) and the *warming* current is applied for 1 sec. to melt the agar. The needle is raised and the tip is moved, as shown by the arrows, to avoid possible damage to the organism, to the other side of the field where the process is repeated. The marking needle is removed and the organism is re-located with the 4 mm. objective. The stage verniers are read and the block is returned to the original reading of the lateral movement, when the edge of the inoculum is found.

Each selected organism is treated in the same way, but each is taken from a different point on the edge of the inoculum and to a greater distance from the inoculum. Finally, a line of pits marks the limit of the inoculated area. The same microneedle can be used if kept in a sterile tube when withdrawn from the cell.

(5) *Cultivation of the isolated organisms*

Method 1. The entire block, which must contain the requisite nutrients, is incubated in a sterile Petri dish with wads of moist cotton wool to maintain the humidity. The dishes must be insulated from the warm floor of the incubator, either by suspension with cords or by cork insulation. Otherwise marked drying of the agar will occur. Every 2 hr. the agar is cooled (to avoid condensation on the objective) and examined under the Perspex cell on the same mechanical stage. The organisms are located (i) by the stage verniers; (ii) by the locating pits, which move with the organisms during drying and distortion of the block. Viable organisms usually appear as microcolonies after 8 hr. When the colony contains about thirty organisms, the sterile marking needle is lowered into the colony under observation with the 16 mm. objective, is raised and inoculated to a solid medium either by pressing the tip lightly into the surface, or by encircling the tip with a loopful of sterile broth, which washes off many organisms and is spread on the medium.

This method originated from that of Ørskov (1922), when random-smeared blocks were used and it was essential to watch the development of colonies from selected organisms to avoid contamination from adjacent organisms. The method is tedious and now unnecessary except for observation of the mode of fission of the organisms, or when separation of the members of the clone arising from the organism is required (Zelle, 1951; Zelle & Lederberg, 1951), for both of which a heated stage is desirable. The method is unsuitable for anaerobes.

Method 2. The agar block is cut immediately to the left of the line of pits, thus separating the inoculated area, using a small knife made by hammering out thick platinum wire (20 s.w.g.) mounted in a needle-holder. Each pair of pits, with the organism, is cut out in turn with the sterile knife and transferred as a 3 mm. cube of agar to the surface of a 'slope' of solid medium. For an aerobe, the cube is laid with the organism exposed. The pits can be seen and the developing colony observed to arise between them after incubation, if the organism is viable, with a hand-lens or dissecting microscope. For an anaerobe, the organism or spore is trapped between the cube and the medium to facilitate anaerobiosis. Growth appears at the edges of the cube face after incubation in the anaerobic jar.

Method 3. The block is dissected and each cube is transferred to a tube of fluid medium containing the required nutrients. This method should be avoided when handling organisms (e.g. staphylococci) not readily distinguishable from dust contaminants, which may occasionally gain access to the medium during the manipulation of the tubes. This is a good method for anaerobes.

The risk of dust contamination is very slight, provided that draughts are avoided and that the manipulations during which the agar block is momentarily exposed to the air are done deftly and quickly. Several isolations are always made on each of a number of blocks to ensure the selection of several viable organisms and the resulting growths are carefully compared.

A modified technique using a micromanipulator to expedite the isolations

The chief delay is in the replacement and recentring of the microneedle after each use of the marking needle. This is eliminated by mounting the marking needle in a micromanipulator on the right-hand side of the observing microscope. It enters by a second opening in the right-hand end of the cell and, with the long-working-distance attachment, can be kept in position close to the microneedle and below the cover-glass during the isolations. Both needles are centred once only for each agar block.

Using the Singer micromanipulator (Barer & Saunders-Singer, 1948), the marking needle can be mounted in a rocking device held in the micromanipulator and operated by a pneumatic capsule and rubber bulb. The micromanipulator then controls the marking needle in the horizontal plane only and the rocking device causes the warm needle to dip into the agar to a fixed depth and to return, thus forming the pits very rapidly and uniformly.

A device for making microneedle tips in a standard microscope

Of methods for making needle tips under microscopic observation, those of Schouten (1907, 1934), Péterfi (1924) and Thaysen & Morris (1947) require both right- and left-handed micromanipulating devices and that of Hilson (1952) a single instrument. The 'microforge' of de Fonbrune (1937) is self-contained and very complete, but costly. The present method (Johnstone, Crofts & Evans, 1950) requires no micromanipulator, the two attachments for the microscope require only simple lathe work for their construction and the movements of the microscope supply all necessary adjustments except for rotation of the small stage carrying the microneedle.

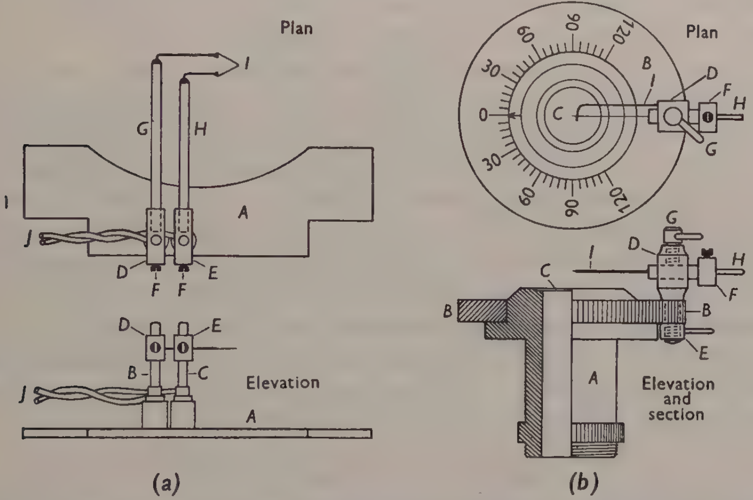


Fig. 5. A device for making micro-instruments: (*a*) plan and back elevation of stage attachment; (*b*) plan and elevation (with partial section) of substage attachment.

(1) *The substage attachment* carries the microneedle on a small stage mounted on a pillar above the microscope stage, but screwed into the substage. Raising and lowering of the needle are therefore effected by the focusing movement of the substage and centration by its centring movement. An adaptor is fitted into the condenser mount carrying R.M.S. female thread. A hollow pillar *A* (Fig. 5*b*) with R.M.S. male thread at its lower end screws into the adaptor, passes upwards through the mechanical stage and carries the rotating stage *B* and a finely ground cover-glass *C*. Light from the microscope mirror passes up the pillar and illuminates the cover-glass, thus providing a diffused

illumination for the needle. The pillar should be matt black inside and the bearing for the stage should be carefully fitted.

At its edge, the stage carries a rotating brass turret *D*, with clamping nut *E*. The steel needle-holder *F* is adjustable in the turret, being locked by the screw *G*. The microneedle handle *H* is lightly clamped in the holder by a small screw and is adjusted so that the needle shaft is supported almost at its end by the crutch *I* fitted to the turret. The crutch is made by grinding away a portion of the eye of a no. 9 crewel needle, bent to shape, and overcomes the flexibility of the shaft during the pull exerted by the heating element. Centration of the tip is effected roughly by the adjustments of the turret and needle holder and finally by the substage centring movement. Rotation of the stage *B* enables the needle tip to be presented at any angle to the direction of pull of the heating element, thus varying the inclination of the tip. (The stage *B* may be graduated in 6° divisions as illustrated, reading against a zero mark on the central pillar, to enable exact reproduction of a given needle tip.)

(2) *The stage attachment* carries a platinum heating element on a base-plate fitting into the mechanical stage, thus giving motion in two dimensions to the element by operating the stage controls. A brass plate *A* (Fig. 5*a*) 3 × 1 in. forms the base, being cut away in front to allow for the pillar of the substage attachment. The base illustrated fits the attachable 'Service' mechanical stage (Watson). Steel pillars *B* and *C* are mounted in brass sockets, with insulating sleeves, on the base-plate and brass blocks *D* and *E* are clamped on the pillars by locking screws *F*, enabling the element to be adjusted for height. Steel rods *G* and *H* project forwards from the blocks above the rotating stage and hold a platinum heating element *I*, made as described for the marking needle, but with a simple V-shaped element (36 s.w.g.). Electrical connexions are made to the bases of the pillars with fine silk-covered flexible wire *J* from a 3 V. transformer, with a rheostat and a foot-switch in the circuit.

Protection from mechanical damage and from over-heating can be given to the 16 mm. objective by fitting a brass cell to the front of the objective, with a protecting cover-glass and a connexion to a filter pump, by which means a current of cool air is drawn across the front lens.

A method for making simple needle tips

With the microscope stage horizontal, the tip of the shaft is centred in the field of the 16 mm. objective and the heating element is brought almost to touch the shaft and is focused accurately. The needle shaft is brought into sharp focus by the substage rack and pinion, being then inclined by rotation of the stage to give the required tip angle (normally 30° with the horizontal). The stage must be turned so that the traction from the element forces the needle shaft into the crutch (Fig. 6*a*), thus eliminating the flexibility of the long shaft. The foot-switch is closed and the rheostat adjusted to bring the element to red heat. On bringing the point of the element into contact with the end of the shaft, the glass fuses on to the platinum and, on withdrawing the element quickly, the glass is drawn out to form the needle tip. By controlling (*a*) the temperature of the element, (*b*) the rate of pull, and (*c*) the time at which the current is cut off by the foot-switch, the length, steepness of taper and terminal diameter of the tip are controlled. A short tip tapering rapidly is ideal as flexibility in the tip must be avoided. It is a great advantage to prepare the tip with a double bend (Fig. 6*b*), thus giving the needle shaft good clearance above the agar surface, if the working distance will permit.

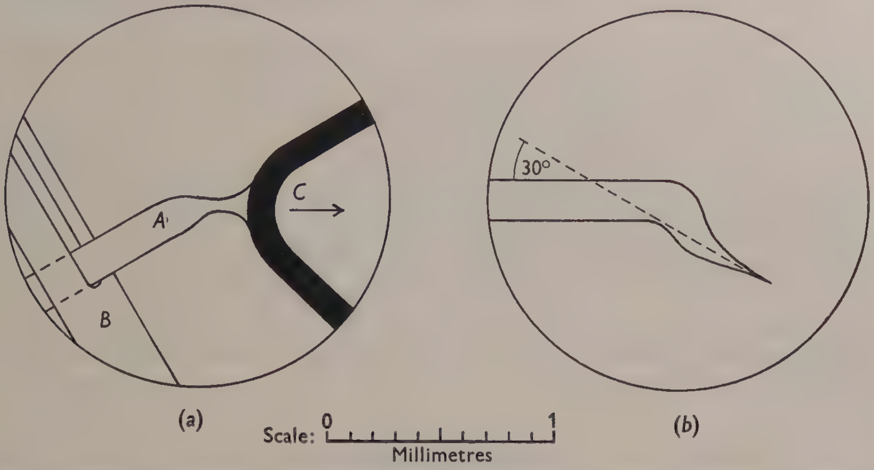


Fig. 6. Making a microneedle tip: (*a*) the end of the shaft *A*, held in the crutch *B*, fused on to the platinum element *C* which is moved as shown by the arrow; (*b*) the completed tip showing the double bend, steep taper and angle of inclination (30°). Image reversed: 16 mm. objective in both (*a*) and (*b*).

Hooks and loops are formed from straight tips by stroking the end of the glass filament with the platinum element at a low temperature until the required curvature is obtained. Directions for making many micro-instruments are given by de Fonbrune (1949), for which the above attachments can be used. When traction by gravity is required (as in the making of micropipettes), the microscope is used in the horizontal position, with the inclined eyepiece turned upwards. An air-blast, as described by de Fonbrune, is not essential for the simpler operations, but can be added as a separate attachment if required.

REFERENCES

Barer, R. & Saunders-Singer, A. E. (1948). A new single-control micromanipulator. *Quart. J. micr. Sci.* 89, 439.

de Fonbrune, P. (1937). *Micromanipulateur pneumatique et micro-forge pour la fabrication des micro-instruments.* Société Industrielle d'Imprimerie, 10 rue Vallier, Levallois-Perret, Paris.

de Fonbrune, P. (1949). *Technique de micromanipulation.* Paris: Masson et Cie.

Goldie, W., Gordon, M. & Johnstone, K. I. (1948). Mutation in a single-cell culture of *Corynebacterium diphtheriae*. *J. Path. Bact.* 60, 369.

Hilson, G. R. F. (1952). A simple and inexpensive microforge. *J. gen. Microbiol.* 7, 175.

Johnstone, K. I. (1943). A simple technique for the cultivation of organisms from single cells. *J. Path. Bact.* 55, 159.

Johnstone, K. I., Crofts, J. E. & Evans, D. G. (1950). Single cell culture of *Cl. welchii* type A morphologically changed by penicillin. *Brit. J. exp. Path.* **31**, 562.

Koblmüller, L. O. & Vierthaler, R. W. (1933). Über ein Gerät zum Isolieren von Keimen auf der Oberfläche fester Nährböden ('Plattenmanipulator'). *Zbl. Bakt.* (1. Abt. Orig.), **129**, 438.

Ørskov, J. (1922). Method for the isolation of bacteria in pure culture from single cells and procedure for the direct tracing of bacterial growth on a solid medium. *J. Bact.* **7**, 537.

Péterfi, T. (1924). *Mikrurgische Methodik. Abderhalden, Handbuch der biologischen Arbeitsmethoden*, Abt. 5, Teil 2, Heft 5, 479.

Pulvertaft, R. J. V. (1952). The effect of antibiotics on growing cultures of *Bacterium coli. J. Path. Bact.* **64**, 75.

Schouten, S. L. (1907). Methode zur Anfertigung der gläsernen Isoliernadeln, gehörend zu dem Isolierapparat für Mikroorganismen. *Z. wiss. Mikr.* **24**, 258.

Schouten, S. L. (1934). Der Mikromanipulator. *Z. wiss. Mikr.* **51**, 421.

Thaysen, A. C. & Morris, A. R. (1947). The preparation of microtools for the micromanipulator. *J. gen. Microbiol.* **1**, 221.

Zelle, M. R. (1951). A simple single-cell technique for genetic studies of bacteria. *J. Bact.* **61**, 345.

Zelle, M. R. & Lederberg, J. (1951). Single-cell isolations of diploid heterozygous *Escherichia coli. J. Bact.* **61**, 351.

(*Received* 1 *April* 1953)

LEWIN, J. C. (1953). *J. gen. Microbiol.* 9, 305–313.

Heterotrophy in Diatoms

BY JOYCE C. LEWIN*
Osborn Botanical Laboratory, Yale University, New Haven, Conn., U.S.A.

SUMMARY: Forty-two bacteria-free cultures of pennate diatoms from fresh water or soil were tested for ability to grow heterotrophically with glucose as the sole carbon source. Thirteen of these isolates proved capable of growth under these conditions. They comprised seven isolates of *Navicula pelliculosa*, five of other different species of *Navicula*, and one of *Nitzschia* (?) *fonticola*.

One isolate of *Navicula pelliculosa* was tested for its ability to grow heterotrophically on any one of sixty organic compounds. Only glucose, glycerol, and fructose were found to be effective in supporting growth. Glucose was metabolized both in the dark, and in the light in the absence of CO_2. Glycerol and fructose were not utilized in the dark, but supported growth in the light in the absence of CO_2.

There is apparently no previous record that any chlorophyllous diatom has been cultivated successfully in the dark. Miquel (1892) found that certain species of diatoms which he obtained in pure culture would not develop either in the dark or in semi-darkness on organic nutrient solutions, although they retained the power of proliferation when returned to the light after several months in darkness. Benecke (1900) observed no increase in numbers in contaminated cultures of pigmented diatoms maintained in the dark, either in hanging drop cultures or in mass cultures with a rich organic nutrient. Karsten (1901), working with impure diatom cultures, determined growth by making daily cell counts. After the cells had been placed in the dark on a medium containing glycine and glucose, he observed three cells of *Navicula perpusilla* to increase to nineteen in 6 days, and seven cells of *Nitzschia closterium* to increase to eighteen in 3 days. Later workers have been inclined to discredit Karsten's observations because he worked with impure cultures, and because he did not let his experiments run for a long enough period to establish conclusively that the organisms were capable of using an external substrate for growth. Karsten also reported that *Nitzschia palea*, unlike the diatoms previously mentioned, showed only an occasional cell division in darkness. He observed that eleven cells increased to fourteen cells in 2 days, and no further growth was noted after 10 days. Richter (1906) reported that pure cultures of *Navicula minuscula* showed no significant development after a month in the dark on a mixture of glycerol, asparagine and glucose, but considered that slight growth took place in his pure cultures of *N. palea* during the first day in darkness, in agreement with Karsten's observations on the same organism. Meinhold (1911) and Treboux (1905) were unsuccessful in obtaining growth of diatoms in the dark.

Although he did not specifically attempt to obtain growth of *N. palea* in the dark, von Denffer (1947) observed that one cell division could occur in darkness when the cells were well filled with fatty reserves. Such cells were able to divide even in distilled water. On the other hand, fat-free cells, obtained by previous starvation in darkness for 8 days, were not capable of dividing in the dark even in the presence of organic nutrient. Von Denffer also observed that the reserves of fat were almost completely utilized in the course of the one cell-division in the dark, and was of opinion that the cell divisions reported for *N. palea* by Karsten and by Richter were also a result of utilization of intracellular fat. Barg (1943), working with impure cultures of the colourless, and therefore heterotrophic, diatom, *N. putrida*, noted by microscopic observation that glucose, glycerol, oleic acid, sucrose, or raffinose stimulated the production of oil droplets within the cells, but made no mention of any cell multiplication in the presence of these carbon sources. According to Pringsheim (1951), only protein hydrolysates support growth of *N. putrida*, whereas glucose and acetate when present by themselves are not utilized. Pringsheim (1951), in noting that none of the coloured diatoms has been grown heterotrophically, stated that if such heterotrophic species exist they should be looked for among the small species of *Navicula* and *Nitzschia* often to be found in habitats rich in decaying organic matter. It is in fact among such diatoms that heterotrophic forms have been found in the present study. This paper deals with the successful heterotrophic cultivation of several small species of pennate diatoms, and a more intensive investigation made with *Navicula pelliculosa* (Bréb.) Hilse.

---

* Present Address: Maritime Regional Laboratory, Halifax, Nova Scotia, Canada.

EXPERIMENTAL

Methods

Medium. In all cases the mineral medium used contained the following concentrations of salts: K_2HPO_4, 0·02% (w/v); $MgSO_4.7H_2O$, 0·02% (w/v); $Ca(NO_3)_2.4H_2O$, 0·1% (w/v) in distilled water. Traces of B, Mn, Zn, Cu, Mo, and Fe, were added. Silicon was added as potassium silicate ('Solution silicate of potash', Eimer & Amend, New York) to establish a soluble silicon concentration of 1–5 mg. Si/l. before the addition of agar. 1·0% Bacto-Difco agar was used for the preparation of plates and slopes.

The organic compounds to be tested were incorporated in the above medium. Sterilization was usually carried out by autoclaving at 15 lb./sq.in. for 15 min. Seitz filtration was used for those compounds which decompose on autoclaving.

Illumination. Petri plates or test tubes (sloped on racks) were placed under constant illumination of 225 ft.c. provided by 'Daylight' fluorescent tubes.

Temperature. All cultures were grown at 23°.

Gas-phase. In those cases where a CO_2-free atmosphere was required the tubes were enclosed in a glass desiccator above 30% (w/v) KOH, the cover being sealed with vaseline.

General survey

Forty-two bacteria-free cultures of fresh-water diatoms were obtained in the following manner. Samples of water from ponds and ditches, and suspensions of soil, were streaked on agar plates containing only mineral supplements, and were incubated under constant illumination. By the use of routine bacteriological streaking techniques a number of cultures of diatoms were isolated. Tests for the presence of bacteria were carried out by transfers into media containing glucose and Tryptone, and by microscopic examination. Each of these isolates was tested for its ability to grow in the dark on agar media containing minerals, supplemented with 0·5 % glucose. The results are summarized in Table 1, and the heterotrophic species are illustrated in Plate 1.

Table 1. *Diatom isolates tested for heterotrophic growth*

Species	No. of isolates	No. of isolates capable of unlimited growth in the dark on glucose
Nitzschia palea (Kütz) W. Smith or *N. Kützingiana* Hilse	27	0
Nitzschia ? fonticola Grun.	1	1
Hantzschia amphioxys (Ehr.) Grun.	1	0
Gomphonema parvulum Kütz	1	0
Navicula pelliculosa (Bréb.) Hilse	7	7
Navicula minima Grun.	1	1
Navicula spp. (different)	4	4

The seven isolates of *Navicula pelliculosa* were obtained from various localities: four from Connecticut garden soil, one from Connecticut pond water, one from a public fountain in Connecticut, and one from snow from Alaska. Although minor cultural differences between the various strains were observed, it is of interest that all the isolates of this species could grow in the dark on glucose. Since *N. pelliculosa* showed such good growth under these conditions, special attention was given to this species, and a more thorough investigation of heterotrophic growth was carried out with the isolate obtained from Clark's Pond near New Haven, Conn., U.S.A.

Heterotrophic nutrition of Navicula pelliculosa

Survey of organic carbon sources. The normal mode of nutrition of autotrophic algae is photosynthesis, i.e. the fixation of CO_2 using energy derived from light. Conditions for heterotrophy may be obtained in the laboratory by eliminating either light or CO_2 so that no photosynthesis can take place, and supplying an organic carbon source that the organism can use for growth. In order to determine what carbon sources might be utilized by *N. pelliculosa* for growth in the absence of light or CO_2, a large number of organic compounds were tested individually. Each of these compounds was incorporated with minerals into agar slopes. Tubes so prepared were inoculated and then incubated (*a*) in air in light; (*b*) in air in darkness; and (*c*) in CO_2-free air in light. Although one could not be certain that the KOH removed all traces of CO_2, the supply of CO_2 was insufficient for photosynthesis, and no growth was ever obtained on mineral media under these conditions. Therefore, any carbon source which supported growth was effective at a CO_2 tension considerably below that necessary for photosynthesis. The compounds tested in such experiments are listed in the Appendix, with the concentrations employed and explanations where necessary.

Of the sixty compounds tested in this survey, only three were found to support heterotrophic growth of *N. pelliculosa*. Glucose was effective both in the dark, and in light in the absence of CO_2. Glycerol or fructose supported growth in absence of CO_2 only in light; neither compound was utilized in the dark. The results are summarized in Table 2. It is thus apparent that, although the organism normally grows photosynthetically, it can also grow on glucose in the complete absence of photosynthesis. For utilization of glycerol or fructose, light but not CO_2 is necessary; it cannot be stated definitely whether all photosynthetic processes are excluded in these last cases.

Table 2. *Carbon compounds which supported heterotrophic growth of* N. pelliculosa

	Autotrophy	Heterotrophy	
	Light ($+CO_2$)	Light ($-CO_2$)	Dark ($+CO_2$)
Control	++	−	−
Glucose	++	+	++
Glycerol	++	+	−
Fructose	++	+	−

Glucose utilization. In order quantitatively to compare growth rates and total growth on various glucose concentrations in the light ($+CO_2$ and $-CO_2$) and in the dark, colorimetric determinations were made of the amount of pigment extractable from cells on the surface of each agar slope. For this purpose, 10 ml. acetone were used per tube, the extraction being carried out in darkness at room temperature, with occasional shaking, for 1 hr. The extract was then decanted and its optical density determined by means of a Klett colorimeter with a ruby glass filter no. 66.

Six tubes of each glucose concentration were set up under each of the three conditions described on p. 307. On each day when growth was to be measured, one tube of each set was sacrificed for pigment determination. Care was taken in the preparation and inoculation of the slopes to insure maximum uniformity within the sets, in order to minimize the individual variation between tubes. Growth of *N. pelliculosa* in darkness on glucose, as measured by colorimetric determination of the pigment extracted, is given in Table 3. It can be seen that as little as 0·0073% glucose was enough to support some growth of *N. pelliculosa*, but with increasing glucose concentration growth was increased. A glucose concentration between 0·2 and 2·0% was apparently optimal for 35 days' growth in the dark.

Fig. 1 (*a*, *b*, *c*) illustrates growth with and without glucose (1·85%) in (*a*) light $+CO_2$, (*b*) darkness $+CO_2$, (*c*) light $-CO_2$. Essentially comparable results were obtained with other glucose concentrations. There was no growth in the absence of glucose, either in the dark or in light $-CO_2$. There was a shorter lag in light than under the two heterotrophic conditions. Since cell

Table 3. *Growth of* N. pelliculosa *in the dark on glucose*

(Relative growths were measured in terms of pigment production)

Glucose concentration (%)	Days after inoculation 12	15	19	27	35
	Relative growth (Klett colorimeter units)				
0	0	0	0	0	0
0·0014	0	0	0	2	8
0·0073	5	12	9	10	14
0·036	11	21	36	37	48
0·184	13	47	59	55	90
0·92	25	57	67	69	92
1·85	24	48	69	75	83
3·7	16	33	45	61	66
7·5	0	0	0	0	5

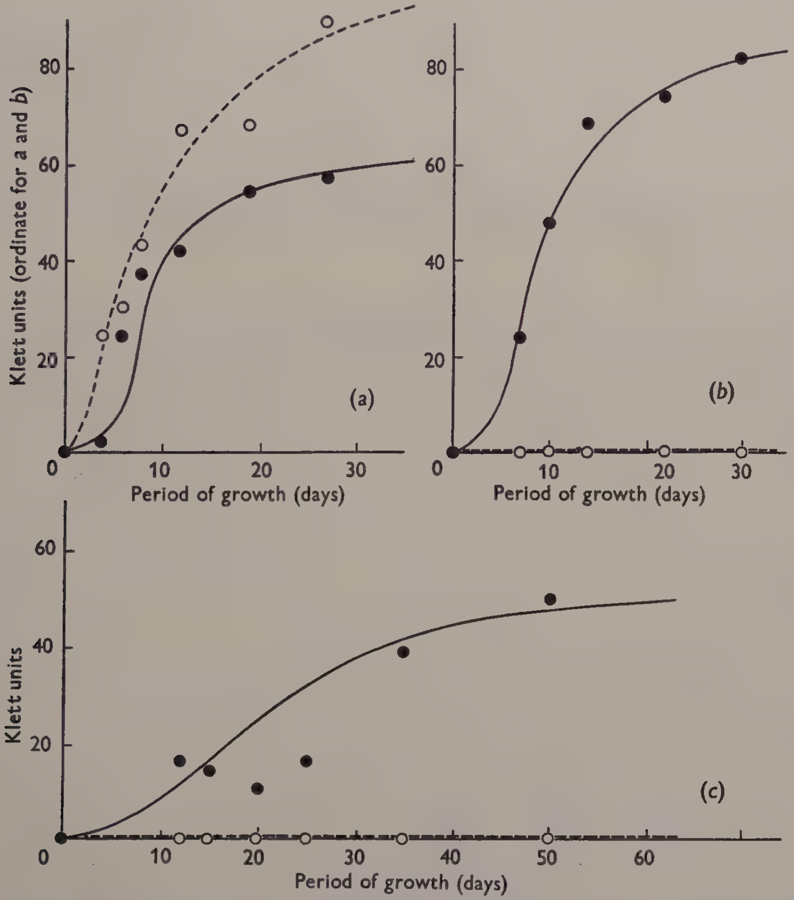


Fig. 1. Growth of *N. pelliculosa* (as measured by optical density of pigment extract). (*a*) in light $+CO_2$; (*b*) in darkness $+CO_2$; (*c*) in light $-CO_2$. With 1·85 % glucose, ●; without glucose addition, ○.

pigments tend to bleach in light more rapidly in the presence of glucose, growth in light (as measured by pigment density) is apparently poorer on glucose than on minimal medium. Following the lag phase, the growth rates on glucose $+CO_2$ in light or darkness were similar, but growth in light $-CO_2$ proceeded much more slowly, possibly indicating a requirement for CO_2 independent of direct photosynthesis.

Glycerol and fructose utilization. Since glycerol supported growth in light in absence of CO_2, but not in the dark, it was considered possible that glycerol and some other compound might together permit growth in darkness. Consequently, in a series of experiments a number of compounds such as sugars, fatty acids, ATP, Tryptone, casein hydrolysate, and various concentrations of inorganic phosphate (0·02% to 0·1% K_2HPO_4) were tested as supplements to glycerol media. (Some of the combinations are incorporated in the Appendix.) In all cases the results were negative. Glycerol, alone or supplemented with other compounds, would not support growth in darkness.

An attempt was made to obtain utilization of fructose in the dark by adding it in various concentrations to media containing 0·002% glucose. It was thought that this small amount of glucose might act in a coupled reaction to provide energy for any initial phosphorylation which might be necessary before fructose oxidation could get under way. There was no growth over and above that supported by 0·002% glucose alone at any of the fructose concentrations tested. It thus appears that light is essential for the heterotrophic utilization of fructose and glycerol by *N. pelliculosa*.

DISCUSSION

It is not surprising that certain species of chlorophyllous diatoms are able to grow heterotrophically. In any group of algae which occupy as wide a range of habitats as do the diatoms, there are almost certain to be those which can utilize organic carbon compounds of the environment for growth in darkness. However, since their rate of growth would probably be considerably slower than that of many obligate heterotrophs, such algae cannot readily be isolated by simple enrichment techniques, and facultative heterotrophy can only be demonstrated when pure cultures have been obtained (see Allen, 1952).

Although an organism may possess the ability to metabolize an organic compound, it does not follow that the energy or the intermediates so produced would necessarily be available for growth. For instance, although *N. pelliculosa* is unable to utilize lactic, acetic, pyruvic, citric, or succinic acid for heterotrophic growth, each of these substrates can stimulate respiration of this diatom when tested in Warburg manometric experiments (Lewin, unpublished). The organism can thus apparently metabolize such substrates but cannot utilize them for growth. The total free energy from oxidation of lactic acid is not markedly different from that liberated in glucose oxidation, yet lactic acid, unlike glucose, cannot serve as a substrate for growth in darkness. It would therefore seem that the critical factor here is not energy, but rather some essential intermediate (Y) which may be formed during glucose oxidation. Possibly this intermediate can be formed heterotrophically in the light when either fructose or glycerol is supplied to the cells. However, compounds such as hexose diphosphate, fructose-6-phosphate, phosphoglyceric acid, and glucose-1-phosphate, which one might have expected to be convertible into the intermediate (Y), did not support heterotrophic growth at all.

The fact that light is required for the heterotrophic utilization of fructose or glycerol by *N. pelliculosa* merits further consideration. It is possible that fructose or glycerol may be first converted by the cells to some intermediate (X). Light may then act by converting (X) into (Y), a hypothetical compound required for growth.

$$\text{Glycerol or fructose} \longrightarrow (X) \xrightarrow{\text{Light}} (Y) \longrightarrow \text{Growth.}$$

If (X) were CO_2 then light would be acting in a normal photosynthetic fixation of CO_2. However, it is doubtful whether CO_2 itself is the intermediate in the photochemical process, since lactic acid, which can be oxidized by *N. pelliculosa* to CO_2 (Lewin, unpublished), cannot support growth under conditions where glycerol or fructose is effective. Some compound other than CO_2 would probably be involved if a scheme such as that presented above were operative. Interpretation of this phenomenon is rendered more difficult by the fact that glycerol or fructose shows only slight stimulation to respiration when tested in Warburg manometric experiments (Lewin, unpublished).

Acknowledgment is made to Dr R. A. Lewin for aid in the isolation of the diatoms used in this study, and for the preparation of the electron-micrographs. Some of the material included in this paper was presented as part of a Ph.D. thesis submitted to Yale University in 1952. The author is indebted to Dr E. G. Young, Maritime Regional Laboratory, Halifax, Nova Scotia, for critical assistance in the preparation of this paper.

APPENDIX

Carbon compounds tested for ability to support heterotrophic growth of Navicula pelliculosa

(The figures in brackets following the concentrations tested are the lowest concentrations (%) which were inhibitory to growth in light.)

Compound	Concentrations tested (%)*
Organic acids:	
citric	0·1
crotonic	0·1
fumaric	0·1
α-D-galacturonic	0·5, 0·1 (0·5)
gluconic	2·5–0·02 (2·5)
D (+) glucuronic acid	0·5, 0·1 (0·5)
D (+) glucuronic lactone	0·5, 0·1 (0·1)
glycollic	0·75–0·001
α-ketoglutaric	0·1
lactic	0·5, 0·1, 0·01 (0·5)
malic	0·1
oxalic	0·1, 0·01
pyruvic	0·5, 0·1, 0·02† (0·1)
succinic	0·1
formic	4·4–0·0004‡ (1·1)
acetic	0·5–0·0004‡ (0·05)
propionic	0·5–0·0004‡ (0·01)
butyric	0·5–0·0004‡ (0·01)
valeric	0·5–0·0004‡§ (0·01)
stearic	0·5–0·004§ (0·01)
oleic	0·5–0·004§ (0·004)
lauric	0·5–0·004§ (0·1)
myristic	0·5–0·004§ (0·1)
Organic esters:	
ethyl acetoacetate	1·5, 0·15 (0·15)
ethyl malonate	1·5, 0·15 (1·5)
ethyl oxalate	1·5, 0·15 (0·15)
ethyl succinate	1·5, 0·15 (1·5)
Alcohols:	
ethanol	0·02
ethyleneglycol	0·5, 0·1, 0·02
glycerol	2·5–0·15
mannitol	0·5
Hexoses, etc.:	
D-galactose	0·5
D-glucose	15–0·0014 (15·0)
D-fructose	4·0–0·06
α-methylglucoside	0·5
Pentoses:	
L-arabinose	0·5, 0·1
D-arabinose	0·5, 0·1
fucose	0·5, 0·1
Pentose:	
rhamnose	0·5
sorbose	0·5
xylose	0·5, 0·1
Disaccharides:	
cellobiose	0·5, 0·1
lactose	0·5, 0·1
maltose	0·5
melibiose	0·5, 0·1
sucrose	0·5
trehalose	1·0, 0·05, 0·01
Trisaccharides:	
D-melezitose	0·5, 0·1
D-raffinose	0·5, 0·1
Phosphate compounds:	
glucose-1-PO_4	0·2, 0·1‡
adenosine triphosphate	0·005‡
Na glycerophosphate	0·5‡
hexose diphosphate	0·5, 0·05‡
phosphoglyceric acid	0·5, 0·1
fructose-6-phosphate	0·5
Nitrogen compounds:	
DL-alanine	0·5, 0·05
L-aspartic acid	0·5, 0·1, 0·05
L-glutamic acid	0·5, 0·1, 0·05
glycine	0·5, 0·1, 0·05
Various:	
soluble starch	0·5
Tryptone (Difco)	0·4–0·05
yeast extract (Difco)	0·1
casein hydrolysate	0·12
corn oil	0·2‖ (0·2)
'Crisco' (hydrogenated vegetable oil)	0·2‖ (0·2)
milk	0·2‖ (0·2)
soy bean oil	0·2‖ (0·2)
butter	0·2‖ (0·2)
olive oil	0·2‖ (0·2)
vitamin mixture	
hydrolysed products of dried sheath material extracted from *N. pelliculosa*	2·5, 0·25, 0·025 (0·25)

* When a range of concentrations was tested only the extremes are given.

† The 0·5% and 0·02% concentrations were tested on Petri plates at various pH levels using 'Penassay' paper pads.

‡ Tested both in the presence and absence of 0·5% glycerol.

§ Tested in the presence of 0·5% glycerol + 0·006% Tween 80.

‖ Tested with 0·025% Tween 80.

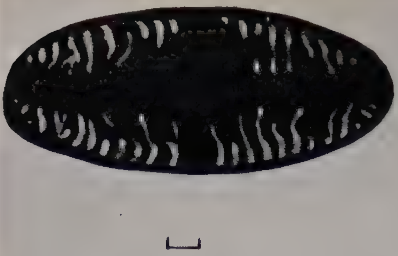


Fig. 1

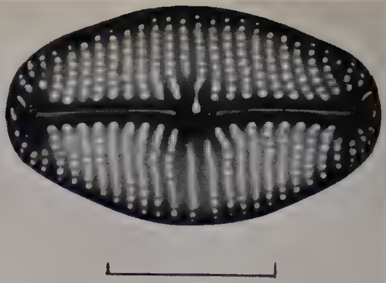


Fig. 2

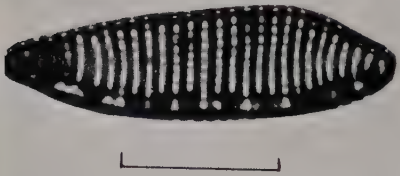


Fig. 3

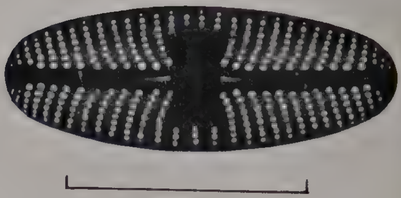


Fig. 4

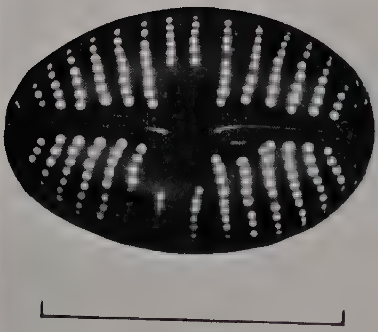


Fig. 5

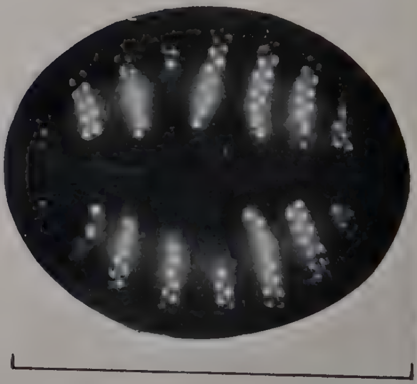


Fig. 6

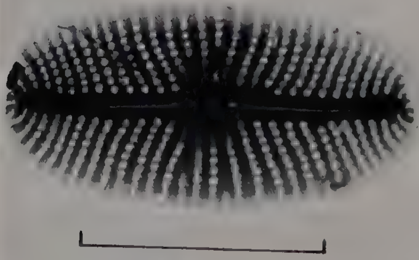


Fig. 7*a*

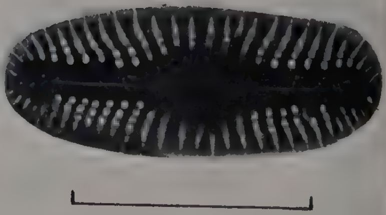


Fig. 7*b*

J. C. Lewin—Heterotrophy in diatoms. Plate 1

REFERENCES

ALLEN, M. B. (1952). The cultivation of *Myxophyceae*. *Arch. Mikrobiol.* **17**, 34.

BARG, T. (1943). Über den Fettgehalt der Diatomeen. *Ber. dtsch. bot. Ges.* **61**, 13.

BENECKE, W. (1900). Über farblose Diatomeen der Kieler Föhrde. *Jb. wiss. Bot.* **35**, 535.

KARSTEN, G. (1901). Über farblose Diatomeen. *Flora*, **89**, 404.

MEINHOLD, T. (1911). Beiträge zur Physiologie der Diatomeen. *Beitr. Biol. Pfl.* **10**, 353.

MIQUEL, P. (1892). De la culture artificielle des Diatomées. *Le Diatomiste*, **1**, 93.

PRINGSHEIM, E. G. (1951). Über farblose Diatomeen. *Arch. Mikrobiol.* **16**, 18.

RICHTER, O. (1906). Zur Physiologie der Diatomeen. *S.B. Akad. Wiss. Wien. Abt. I.* **115**, 27.

TREBOUX, O. (1905). Organische Säuren als Kohlenstoffquelle bei Algen. *Ber. dtsch. bot. Ges.* **23**, 432.

VON DENFFER, D. (1947). Die planktische Massenkultur pennater Grunddiatomeen. *Arch. Mikrobiol.* **14**, 159.

EXPLANATION OF PLATE

Electron-micrographs of diatom species capable of heterotrophic growth. Each scale line represents 5 μ.

Fig. 1. ? *Navicula* sp. × 850.
Fig. 2. *Navicula* sp. (section *minusculae*) × 4450.
Fig. 3. *Nitzschia ? fonticola* Grun. × 4330.
Fig. 4. *Navicula minima* Grun. × 6250.
Fig. 5. *Navicula* sp. (section *minusculae ? = N. muralis*) × 8170.
Fig. 6. ? *Navicula* sp. × 10,400.
Fig. 7. *Navicula pelliculosa* (Bréb.) Hilse. (*a*) × 6500 (from Connecticut pond); (*b*) × 6125 (from Alaska snow).

(*Received* 27 *April* 1953)

JEFFERYS, E. G., BRIAN, P. W., HEMMING, H. G. & LOWE, D. (1953). *J. gen. Microbiol.* 9, 314–341.

Antibiotic Production by the Microfungi of Acid Heath Soils

BY E. G. JEFFERYS, P. W. BRIAN, H. G. HEMMING AND D. LOWE

Imperial Chemical Industries Limited, Butterwick Research Laboratories, Welwyn, Hertfordshire

SUMMARY: A study has been made, using a plating technique, of the fungi occurring in acid sandy podsol soils and of their distribution at different levels of the soil profile. The most widespread species, characteristic of this type of soil, included: *Absidia orchidis, Mortierella alpina, Mucor ramannianus, Penicillium frequentans, P. nigricans, P. roqueforti, P. terlikowskii* and *Trichoderma viride.* A number of other species were locally abundant, including *Absidia glauca, Coniothyrium* sp., *Gliocladium* sp. (*roseum* series), *Penicillium citrinum* and *Zygorhyncus moelleri.*

The plate counts of viable fungal units decreased sharply from upper to lower levels, though there was occasionally a secondary maximum in the upper part of the B horizon. Most species were characteristically found most abundantly in upper layers of the A horizon, but *Mucor ramannianus* was notable for its abundance at much lower levels; in several of the profiles examined it was isolated in almost pure culture from the B horizon.

Of the sixty-five species isolated, about half produced antibiotics. Most of these were toxic to fungi and bacteria; only a few cases of specific antifungal or antibacterial activity were encountered. The Phycomycetes differed from other groups in that none produced antifungal substances. The capacity to produce antifungal antibiotics appears to be correlated with the distribution of species; of the widespread and locally abundant fungi, 45 % produced such antibiotics as compared with 15 % of the rare fungi. Nevertheless, certain widespread species (e.g. *Absidia orchidis, Mortierella alpina, Mucor ramannianus, Penicillium roqueforti*) did not produce antifungal antibiotics, so it cannot be considered to be a necessary character of widespread soil fungi. There is some evidence that such widespread non-producers of antibiotics are relatively resistant to the antibiotics produced by other species.

An attempt has been made to group the fungi isolated into Burges' ecological categories of 'sugar-fungi' and 'humus-fungi' on the basis of their capacity to attack and utilize carboxymethylcellulose (CMC). Only 26 % of the fungi were unable to utilize CMC, Phycomycetes being prominent among these. Whereas 35 % of the rare fungi and 54 % of locally abundant fungi were 'sugar-fungi', only 17 % (all Phycomycetes) of the widespread fungi were 'sugar-fungi'.

These results are believed to lend support to the view that antibiotic production is of ecological significance to soil fungi. They also provide information which can form the basis of an experimental search for more direct evidence.

The conception that antibiotic production may be of significance in the relations between soil micro-organisms stems mainly from observations of four kinds: (1) that a high proportion of soil micro-organisms, including fungi, bacteria and actinomycetes, produce antibiotics *in vitro*; (2) that fungi parasitic on the roots or underground stems of plants survive in soil only for a limited time in competition with certain saprophytic organisms; (3) that among the more effective of these saprophytic antagonists are such organisms as the fungus *Trichoderma viride*, whose capacity to produce antibiotics *in vitro* is well-known; (4) that root-parasitic fungi are in general more sensitive to antibiotics than the saprophytic micro-organisms which produce them. These points have recently been dealt with more fully elsewhere (Brian, 1949*a*, 1949*b*; Garrett, 1950, 1951). It has frequently been suggested, therefore, that the basis of the antagonism between soil-saprophytes and root-parasites is, in part at least, the production of antibiotics by the former. This hypothesis has rested mainly on circumstantial evidence and cogent arguments can be advanced against it. This paper reports one aspect of a general investigation intended to produce more direct evidence.

It seemed to us that an approach based on the study of micro-organisms, antibiotics and soils biologically unrelated to one another would not necessarily give information of value. We have therefore sought: (1) to obtain a detailed knowledge of the microflora of a well-defined type of soil and of the capacity of the species concerned to produce antibiotics *in vitro*; the present paper deals exclusively with this aspect and the soil fungi only are considered; (2) to obtain knowledge of the biological and chemical properties of the antibiotics produced by these organisms; this has been dealt with in several publications referred to elsewhere in this paper; (3) to determine the stability of these antibiotics in soils of the type under investigation; results have been presented by Jeffreys (1952); (4) to obtain information bearing directly on the capacity of organisms isolated from the chosen soil to produce antibiotics under soil conditions; preliminary results have been reported by Wright (1952).

The type of soil chosen for study was an acid sandy podsol, such as develops characteristically in this country on Bagshot sand deposits. Such soils are naturally poor and usually bear a heath vegetation; they have little economic value except for forestry. This type was chosen because of some evidence of occasional accumulation of biologically produced substances toxic to fungi (Neilson-Jones, 1941); it has been suggested that these toxins are antibiotics produced by certain fungal species characteristic of the soil (Brian, Hemming & McGowan, 1945; Rayner, 1945). These soils have a well-developed profile, the chemical and physical properties of the soil differing widely in different zones of the top 2–3 ft. Since such physico-chemical differentiation would be expected to result in differences in the microflora at different levels, the soils have been studied in depth as well as at different localities on the same geological formation. Thus, in addition to providing basic material for the other investigations mentioned in the preceding paragraph, this work has provided information on the ecology of the fungi of acid heath soils and affords an interesting comparison with the survey of fungi of Breckland soils recently undertaken by Warcup (1951).

MATERIALS AND METHODS

The soils

Soils were obtained from nine profiles exposed by digging pits of approximately 2 ft. square cross-section and depth of 15–26 in. These were all on soils derived from Bagshot sands, four on Bagshot Heath, Surrey, and five on Wareham Heath, Dorset. The structural and physical characteristics of these profiles are summarized in Tables 2–10; all were highly acid at all levels. The majority of these soils were well-developed podsols but some, as was noted by Neilson-Jones (1941), rested directly on the subsoil without a pan.

Sampling

After exposure of the profile, the surface was picked off with a sterile stainless steel tool, to reduce the possibilities of contamination of the lower layers by soil from upper layers during digging. Fairly large samples (*c.* 200 g.) of soil were then taken from different layers, again using sterile tools, and placed in sterile screw-capped bottles. Dates of sampling were: profiles I–IV, 13 October 1947; profiles V–IX, 29 January 1948.

Isolation of organisms

The conventional plating technique was adopted. Each soil sample was well mixed, a weighed portion of the order of 25 g. was added to 250 ml. sterile water in a flask and shaken well by hand for 15 min. Three further tenfold dilutions of this suspension were made with sterile water; 1 ml. samples of these were used to inoculate the surface of agar plates incubated at 25° for 6 days previously. The moisture contents of the soils were determined, so that the number of colonies appearing on each plate could be related to the numbers of viable units per g. dry soil. In general, eight plates were inoculated with each dilution and were incubated at 25°. Colony counts were made after 6 days. The plates were kept for some time after this and examined from time to time in case new colonies developed.

The medium used for isolation had the following composition: KH_2PO_4, 1·0 g.; $MgSO_4.7H_2O$, 0·5 g.; ammonium tartrate, 2·2 g.; *dl*-malic acid, 5·0 g.; peptone, 1·0 g.; yeast extract (Difco Laboratories Inc., Detroit), 1·0 g.; glucose ('Dextrolact'—Corn Products Ltd., Strand, London, W.C. 2), 20·0 g.; minor element mixture, containing iron, manganese, copper, zinc and molybdenum, as described by Brian, Curtis & Hemming (1947), 1·0 ml.; Rose Bengal, 0·067 g.; agar, 25·0 g.; distilled water to 1 l. This somewhat complex medium was used to ensure that organisms requiring ammonia or amino-nitrogen, or vitamins, were not excluded. The malic acid was included as a result of work which showed that certain organic acids aid the development of some fungi on ammonia-nitrogen media (Brian *et al.* 1947). Preliminary experiments showed that appreciably greater numbers of colonies developed on this medium than on other media recommended for isolation of fungi from soil.

Representative colonies were subcultured on slopes; at the same time a note was made of the frequency of similar colonies, on a simple arbitrary scale (see Tables 2–10). About 950 isolations were made in this way for subsequent examination and identification.

The soils were not all plated immediately after collection, but were stored in a refrigerator until used. The actual dates of plating were: profile I, 14 October 1947; profile II, 28 October 1947; profile III, 12 November 1947; profile IV, 26 November 1947; profiles V and VI, 30 January 1948; profile VII, 9 April 1948; profile VIII, 23 April 1948; profile IX, 30 April 1948.

On each occasion when soils were plated, control plates were poured and handled in every way like the dilution plates except that they received no soil inoculum. Organisms appearing both on the control plates and the soil dilution plates were considered to be contaminants from the laboratory air.

Determination of antibiotic activity

All the representative subcultures were tested by a streak test on agar media (Brian, Curtis, Hemming, Jefferys, Unwin & Wright, 1951) for antagonism to the fungus *Candida albicans* and the bacteria *Staphylococcus aureus, Salmonella typhi* and *Bacterium coli*. A somewhat smaller selection of isolates of each species was grown in liquid media (30 ml. in 100 ml. flasks) and the culture filtrates were assayed from time to time by the *Botrytis allii* spore germination test (Brian & Hemming, 1945) and by *Bacillus subtilis* and *Bacterium coli* cylinder plate assays. The media used were those of Czapek-Dox and Raulin-Thom, each containing 5 % glucose ('Dextrolact'). Some fungi failed to develop on these media, but did so when 0·01–0·1 % 'Difco' yeast extract was added.

Utilization of carboxymethyl cellulose

A medium of the following composition was dispensed in 10 ml. quantities in 100 ml. flasks: ammonium tartrate, 2·0 g.; potassium dihydrogen phosphate, 1·0 g.; magnesium sulphate (hydrated), 0·5 g.; potassium chloride, 0·5 g.; ferrous sulphate, 0·01 g.; sodium carboxymethylcellulose, degree of substitution, 0·45–0·5 mol. ('Cellofas' B—I.C.I. Ltd., Nobel Division, Stevenston, Ayrshire), 10·0 g.; distilled water, 1 l. After autoclaving, replicate flasks were inoculated with the fungi to be tested. Flasks were harvested after 5 and 10 days' incubation at 25° and the culture filtrates were tested for carboxymethylcellulose (CMC) with chlor-zinc-iodide (Gatenby & Beams, 1950). This reagent reacts with CMC as with cellulose. The uninoculated medium gave a dark blue precipitate with chlor-zinc-iodide. After growth of some fungi no colour reaction was obtained, indicating complete degradation of CMC. A reaction similar to that obtained with uninoculated medium was taken to indicate no degradation of CMC and intermediate reactions partial degradation.

CMC was used as a test substrate in preference to cellulose since, being water-soluble, it is more convenient to handle. It was realized that some fungi able to degrade CMC might be unable to degrade cellulose (Siu, 1951). However, of sixty-two strains (33 spp.) found to attack CMC vigorously, fifty-eight strains (32 spp.) were found to attack cellulose in similar tests using cellulose powder (Whatman B quality, chemically prepared, standard grade for chromatography) instead of CMC. For cellulose powder iodine and sulphuric acid were found preferable to replace the chlor-zinc-iodide reagent (Gatenby & Beams, 1950).

RESULTS

Species isolated. The species isolated are listed in Table 1 below.

Table 1. *List of species isolated*

(The data in parentheses denote their capacity to attack carboxymethylcellulose and the number of cultu tested for this activity; these are referred to elsewhere in the text.)

PHYCOMYCETES

Absidia glauca Hagem (CMC −, 2)
A. orchidis (Vuill.) Hagem (CMC −, 2)
Mortierella alpina Peyronel (CMC −, 3)
M. isabellina Oudemans (CMC −, 3)
M. vinacea Dixon-Stewart (CMC −, 1)
Mucor racemosus Fres. (CMC +, 1)
M. ramannianus Moeller (CMC +, 6)
M. varians Povah (CMC +, 1)
Pilaira anomala (Cesati) Schroeter (CMC −, 1)
Zygorhyncus moelleri Vuill. (CMC −, 2)

ASCOMYCETES

Sordaria fimicola (Rob.) Ces. & De Not. (CMC + +, 2)

FUNGI IMPERFECTI

Sphaeropsidales
Coniothyrium sp. 977 (CMC + +, 3)
Phoma spp. 802 (CMC + +, 1), 1015 (CMC + +, 1), 1079 (CMC +, 1)

Melanconiales
Pestalotia sp. 799 (CMC + +, 1)

Moniliales
Aspergillus ruber (Spieckermann & Bremer) Thom & Church (CMC −, 5)
Aspergillus sp. 912 (CMC +, 1)
Cephalosporium acremonium Corda (CMC −, 1)
(*Cladosporium herbarum* Link ex Fr.) (CMC + +, 6)
Gliocladium sp. 650 (*roseum* series) (CMC variable, see p. 335)
Gliomastix convoluta (Harz) Mason (CMC + +, 1)
Oidiodendron griseum Robak (CMC + +, 1)
Papularia sphaerosperma (Pers.) von Höhnel (CMC + +, 1)
Penicillium adametzi Zaleski (CMC −, 2)
P. albidum Sopp (CMC + +, 3)
P. charlesii Smith (CMC + +, 1)
P. chrysogenum Thom (CMC −, 1)
P. citreoviride Biourge (CMC −, 2)
P. citrinum Thom (CMC + +, 3)
P. fellutanum Biourge (CMC +, 1)
P. frequentans Westling, form A (CMC + +, 2)
P. frequentans Westling, form B (CMC + +, 4)
P. godlewskii Zaleski (CMC + +, 2)
P. lanosum Westling (CMC + +, 5)
P. lividum Westling (CMC + +, 2)
P. melinii Thom (CMC +, 4)
P. nigricans (Bainier) Thom, form A (CMC + +, 5
P. nigricans (Bainier) Thom, form B (CMC + +, 8
P. notatum Westling (CMC + +, 1)
P. palitans Westling (CMC + +, 1)
P. paxilli Bainier (CMC −, 1)
P. roqueforti Thom (CMC + +, 3)
P. rubrum Stoll (CMC + +, 1)
P. spinulosum Thom (CMC variable, see p. 335)
(*P. stoloniferum* Thom) (CMC + +, 3)
P. tardum Thom (CMC −, 1)
P. terlikowskii Zaleski (CMC + +, 3)
P. thomii Maire (CMC + +, 3)
P. urticae Bainier (CMC +, 1)
Penicillium sp. 895 (? *nigricans* series) (CMC +, 4)
Penicillium sp. 1006 (*raistrickii* series) (CMC +, 4)
Pullularia pullulans (de Bary & Low) Berkh (CMC −, 1)
Scopulariopsis spp. 909 (CMC −, 1), 1005 (CMC + 1), 1068 (CMC +, 1)
Sporotrichum sp. 1038 (? *roseum* Link) (CMC + +,
Torula spp. 781 (CMC −, 1), 787 (CMC −, 1)
Trichocladium asperum (Corda) Harz (CMC + +,
Trichoderma viride Pers. ex Fr. (CMC + +, 7)
Verticillium sp. 1027 (? *terrestre* (Link) Lindau) (C variable, see p. 335)

Mycelia sterilia
Sclerotium sp. 1077 (CMC −, 2)
'Fungus A' (1066) (CMC + +, 1)
'Fungus B' (1067) (CMC + +, 1)

The following monographs have been used in compiling this list: for *Penicillium*, Raper & Thom (1949); for *Aspergillus*, Thom & Raper (1945); for *Mortierella*, Linnemann (1941); for other Phycomycetes, Zycha (1935) and Naumov (1939).

Species identified to genus only have been given code numbers in the list. A number of species which occurred frequently on control plates and which are believed to be laboratory atmosphere contaminants have not been included. The more important of these contaminant species were several fasciculate penicillia (*Penicillium cyclopium* Westling, *P. expansum* Link, *P. gladioli* Machacek, *P. puberulum* Bainier) and two Aspergilli (*Aspergillus sydowi* (Bain. & Sart.) Thom & Church, *A. repens* (Corda) de Bary). Two other species appeared both on control plates and on soil-dilution plates—*Penicillium stoloniferum* and *Cladosporium herbarum*. Their appearance on the soil-dilution plates was on some occasions so regular that it seemed possible that they did occur in the soil as well as in the laboratory air. They have therefore been included in parentheses in Table 1 to indicate the doubt about their real status. Warcup (1951) also had doubts concerning the status of *C. herbarum*.

A few species remained completely unidentified. Most of these were slow-growing dark-coloured moulds of rare occurrence; they have not been considered further. Two white sterile fungi were of frequent occurrence in some soils and are included in the list of species isolated and referred to as 'fungus A' and 'fungus B'. These are possibly Basidiomycetes, though no clamp-connexions were seen.

Elsewhere in the text it will be shown that in each of the two species *Penicillium nigricans* and *P. frequentans*, two forms could be distinguished on biochemical and cultural characters. In all statistical material in subsequent parts of the text these are counted as distinct species. It is worthy of note that of the 65 species isolated 28 were species of *Penicillium*.

Distribution of fungi in the soil profiles

Total plate counts, which may be regarded as an imprecise measure of the number of viable fungal units in each gram of dried soil, and the species isolated from different levels of each soil profile are shown in Tables 2–10. The abundance of each species at each level is recorded on a +, + +, + + + system.

Plate counts were highest in the surface layers and fell sharply in the lower layers. In some of the profiles which had a well-defined B horizon, there were indications of an increased plate count in the upper, humus-accumulating layer of the B horizon, as compared with that in the leached A_2 horizon immediately above. The number of species isolated was also generally greatest in surface layers, but this fell off much less rapidly in depth than the plate count.

The plate count in surface layers varied considerably from profile to profile. In general, counts were higher where there was a definite surface layer of litter or humus. In profile IX the low numbers of species and the low plate

Table 2. *Characteristics of profile I (Greyspot Hill, Lightwater, Surrey), plate counts (thousands of viable units/g.* D.W.), *and nature and abundance of fungi isolated*

Vegetation. Poor *Calluna* heath. Profile structure: A_1 (0–2 in.), sand containing humus; A_2 (2–8 in.), leached sand; B (8–12 in.), iron pan with flints; C (12–15 in.), yellow sand with flints.

Occurrence of species. + + + = abundant; + + = frequent; + = rare; . = not observed. D.W. = dry weight, F.W. = fresh weight of soil.

Sample no.	Depth (in.)	Horizon	pH	D.W./F.W. (%)	Plate count	*Absidia orchidis*	*Aspergillus ruber*	*Cladosporium herbarum*	*M. racemosus*	*Mucor ramannianus*	*P. charlesii*	*P. chrysogenum*	*P. fellutanum*	*P. frequentans* f. A	*P. frequentans* f. B	*P. roqueforti*	*P. spinulosum*	*P. stoloniferum*	*P. tardum*	*P. terlikowskii*	*Penicillium urticae*	*Phoma* sp. 802	*Sporotrichum* sp. 1038	*Torula* sp. 781	*Trichoderma viride*	Total no. of species
1	0–1	A_1	4·1	89	58	++	.	+	.	+	.	.	.	.	+++	+++	+++	.	.	.	+	.	+	+	++	10
2	1–2	A_1	4·0	92	2	.	+	++	+	++	.	.	.	.	+++	++	++	+	.	+	.	.	.	.	++	10
3	2–4	A_2	4·2	96	1	.	.	++	.	+++	.	.	.	.	++	.	+	.	.	++	.	.	.	.	++	6
4	4–8	A_2	4·3	97	5	.	.	+	.	+++	+	+	.	+	.	.	+	.	+	.	.	+	.	.	.	8
5	8–10	B	4·5	88	2	.	.	++	.	+++	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	3
6	10–12	B	4·5	92	<1	.	+	+	.	++	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	4
7	12–15	C	4·6	96	<1	.	.	++	.	.	.	.	+	.	+	.	+	.	.	+	.	.	.	.	.	5

Table 3. *Characteristics of profile II (Greyspot Hill, Lightwater, Surrey), plate counts (thousands of viable units/g.* D.W.), *and nature and abundance of fungi isolated*

Vegetation. Poor *Calluna* heath. Profile structure: A_1 (0–2 in.), sand containing humus; A_2 (2–8 in.), leached sand; B (8–9·5 in.), iron pan; C (9·5–16 in.), yellow sand.

Occurrence of species. + + + = abundant; + + = frequent; + = rare; . = not observed. D.W. = dry weight, F.W. = fresh weight of soil.

Sample no.	Depth (in.)	Horizon	pH	D.W./F.W. (%)	Plate count	*Cladosporium herbarum*	*Mucor ramannianus*	*Papularia sphaerosperama*	*P. fellutanum*	*P. frequentans* f. B	*P. lanosum*	*P. roqueforti*	*P. spinulosum*	*P. terlikowskii*	*Penicillium* sp. 895	*Trichoderma viride*	'Fungus B'	Total no. of species
1	0–1	A_1	4·0	94	113	.	.	.	+++	+++	+	.	+++	.	+	+++	.	6
2	1–2	A_1	4·1	93	45	.	++	.	.	+++	.	.	.	+++	.	+++	.	4
3	2–4	A_2	4·3	95	18	.	+++	.	.	++	.	.	.	.	.	+++	.	3
4	4–8	A_2	4·5	95	3	+	+++	+	+	+++	.	++	.	.	.	+++	.	7
5	8–9·5	B	4·4	87	7	.	+++	.	.	++	.	.	.	.	+	+++	.	4
6	9·5–12	C	4·4	96	5	+	+++	.	.	+	.	++	.	.	.	+++	++	6
7	12–16	C	4·8	96	6	.	++	.	.	.	.	+	.	.	.	.	.	2

Table 4. *Characteristics of profile III (Dawney's Hill, Brookwood, Surrey), plate counts (thousands of viable units/g.* D.W.), *and nature and abundance of fungi isolated*

Vegetation. Dense *Pteridium* cover. Profile structure: A_0 (0–2 in.), surface litter and humus; A_1 (2–7 in.), sand containing humus; A_2 (7–20 in.), leached sand; B (20–21 in.), dark iron pan.

Occurrence of species. + + + = abundant; + + = frequent; + = rare; . = not observed. D.W. = dry weight, F.W. = fresh weight of soil.

Sample no.	Depth (in.)	Horizon	pH	D.W./F.W. (%)	Plate count	*Absidia orchidis*	*Cladosporium herbarum*	*Gliocladium* sp. 650	*M. ramannianus*	*Mucor varians*	*P. albidum*	*P. citrinum*	*P. frequentans* f. B	*P. godlewskii*	*P. melinii*	*P. nigricans* f. A	*P. nigricans* f. B.	*P. roqueforti*	*P. terlikowskii*	*Penicillium* sp. 895	*Penicillium* sp. 1006	*Scopulariopsis* sp. 1005	*Sordaria fimicola*	*Sporotrichum* sp. 1038	*Trichoderma viride*	*Zygorhyncus moelleri*	Total no. of species
1	0–1	A_0	4·0	74	388	.	.	+++	.	+	.	.	+++	.	+++	.	+	.	.	.	.	+++	+	.	+++	++	9
2	1–2	A_0	4·0	83	720	++	.	.	.	.	+++	+++	.	.	.	+++	.	.	.	.	++	.	.	.	+++	++	7
3	2–7	A_1	4·1	87	4	.	+	.	+++	.	.	.	+	.	+++	+++	.	.	.	.	.	.	.	.	++	+++	7
4	7–10	A_2	4·1	96	<1	.	.	++	.	.	.	.	+	.	+++	.	.	.	.	+	.	.	.	.	.	+	5
5	10–16	A_2	4·4	96	<1	+	.	++	.	.	.	.	.	+	.	.	.	+	.	.	.	.	.	+	.	.	5
6	16–20	A_2	4·3	98	<1	.	.	.	++	.	.	.	.	.	.	+	.	.	+	.	.	.	.	.	+	.	4
7	20–21	B	4·1	83	<1	.	.	.	.	.	.	.	+	+	.	+	.	.	.	.	.	.	.	.	.	.	3

Table 5. *Characteristics of profile IV (Dawney's Hill, Brookwood, Surrey), plate counts (thousands of viable units/g.* D.W.), *and nature and abundance of fungi isolated*

Vegetation. Thick *Calluna* heath with some *Ulex* and *Betula*. Profile structure: A_1 (0–1 in.), sand containing humus; A_2 (1–12 in.), leached sand and gravel; B (12–15 in.), iron pan; C (15–17 in.), sand.

Occurrence of species. + + + = abundant; + + = frequent; + = rare; . = not observed. D.W. = dry weight, F.W. = fresh weight of soil.

Sample no.	Depth (in.)	Horizon	pH	D.W./F.W. (%)	Plate count	*Absidia orchidis*	*Cladosporium herbarum*	*Mucor ramannianus*	*P. adametzi*	*P. albidum*	*P. fellutanum*	*P. roqueforti*	*P. spinulosum*	*P. stoloniferum*	*Penicillium terlikowskii*	*Trichoderma viride*	'Fungus A'	Total no. of species
1	0–1	A_1	4·1	88	140	+ + +	+	.	+ +	.	.	.	+ +	+ +	+ +	+ +	+ +	8
2	1–4	A_2	4·3	84	14	.	.	+ +	+	.	.	+	.	.	+ +	+ +	.	5
3	4–8	A_2	4·3	99	2	+	+	+ + +	.	+	.	.	.	+	.	.	.	5
4	8–12	A_2	4·4	99	7	.	+ +	+ + +	.	.	.	+	.	+	.	.	.	4
5	12–13·5	B	4·3	96	13	.	+	+ + +	.	.	.	.	.	+	.	.	.	3
6	13·5–15	B	4·3	94	6	.	+	+ + +	.	.	+	.	.	.	+	+	.	5
7	15–17	C	4·4	93	1	.	+	+ + +	.	.	.	.	.	+	.	.	.	3

Table 6. *Characteristics of profile V (Bloxworth Heath, Wareham, Dorset), plate counts (thousands of viable units/g.* D.W.), *and nature and abundance of fungi isolated*

Vegetation. Young Scots Pine plantation with *Deschampsia* ground cover with sparse *Pteridium*. Profile structure: A_0 (0–1 in.), surface litter; A_1 (1–4 in.), sand containing humus; A_2 (4–12 in.), leached sand grading imperceptibly into C (12–20 in.), reddish-brown sand.

Occurrence of species. + + + = abundant; + + = frequent; + = rare; . = not observed. D.W. = dry weight, F.W. = fresh weight of soil.

Sample no.	Depth (in.)	Horizon	pH	D.W./F.W. (%)	Plate count	*Absidia orchidis*	*Aspergillus* sp. 912	*Gliocladium* sp. 650	*Mortierella alpina*	*P. albidum*	*P. nigricans* f. A	*P. nigricans* f. B	*P. notatum*	*P. terlikowskii*	*Penicillium* sp. 895	*Phoma* sp. 1079	*Trichoderma viride*	*Verticillium* sp. 1027	'Fungus A'	'Fungus B'	Total no. of species
1	0–1	A_0	4·1	55	830	+ +	.	+ +	+	.	+ + +	.	.	+ +	.	.	+ +	.	+ +	+ +	8
2	1–2	A_1	4·1	85	173	.	.	+	+	+ + +	+ + +	+ +	.	+	.	+ +	+ +	+	+ +	+ +	11
3	2–4	A_1	4·1	89	53	+ +	+ +	+	.	.	+ + +	+ + +	+ +	.	.	.	.	.	+ + +	.	7
4	4–8	A_2	3·9	89	17	.	.	+	+ + +	.	+ + +	+ +	+	.	.	.	+ +	.	+ + +	.	7
5	8–12	A_2	4·1	87	6	+	.	+	.	.	+ + +	.	.	.	+ +	.	+	.	.	.	5
6	12–16	C	4·2	87	<1	.	.	.	.	.	.	.	.	.	.	.	+	.	+	.	2
7	16–20	C	4·1	88	<1	.	.	.	+	.	.	.	.	.	+	.	.	.	+	.	3

Table 7. *Characteristics of profile VI (Bloxworth Heath, Wareham, Dorset), plate counts (thousands of viable units/g.* D.W.*), and nature and abundance of species isolated*

Vegetation. Recently felled plantation of rather poor pine, with *Calluna*, *Ulex* and *Deschampsia* ground cover. Profile structure: A_0 (0–1 in.) peat; A_1 (1–7 in.), dark humus-containing sticky sand with some clay; A_2 (7–13 in.), leached sandy clay grading without pan into C (13–20 in.), banded sand.

Occurrence of species. + + + = abundant; + + = frequent; + = rare; . = not observed. D.W. = dry weight, F.W. = fresh weight of soil.

Sample no.	Depth (in.)	Horizon	pH	D.W./F.W. (%)	Plate count	*Absidia orchidis*	*Cladosporium herbarum*	*M. alpina*	*M. isabellina*	*Mortierella vinacea*	*P. adametzi*	*P. albidum*	*P. frequentans* f. A	*P. lividum*	*P. nigricans* f. A	*P. nigricans* f. B	*P. palitans*	*P. roqueforti*	*P. spinulosum*	*P. stoloniferum*	*P. terlikowskii*	*P. thomii*	*Penicillium* sp. 895	*Scopulariopsis* sp. 909	*Scopulariopsis* sp. 1008	*Sordaria fimicola*	*Torula* sp. 787	*Trichoderma viride*	*Verticillium* sp. 1027	'Fungus A'	'Fungus B'	Total no. of species
1	0–1	A_0	3·9	59	240	+	.	+++	.	.	+++	.	.	++	+++	.	.	+	++	.	+	++	++	+	+	.	.	+++	.	+	+	15
2	1–3	A_1	4·3	74	15	.	.	.	+	.	++	+	.	.	++	.	.	++	++	+	+++	.	++	.	.	++	.	+++	.	+	.	12
3	3–7	A_1	4·1	79	1	.	+	+	.	.	.	+	.	.	.	++	.	+	.	.	.	.	+	.	+	.	+	+	+	.	.	10
4	7–9	A_2	4·1	79	<1	.	.	.	.	.	.	++	.	.	.	.	.	.	.	.	.	.	+	.	.	.	++	.	.	.	+	4
5	9–13	A_2	4·7	87	<1	.	.	.	.	.	+	.	.	.	.	.	+	.	.	++	.	.	+	.	.	.	+	.	.	.	.	5
6	13–17	C	4·7	91	<1	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	2
7	17–20	C	4·5	92	<1	.	+	.	.	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	4

Table 8. *Characteristics of profile VII (Bere Wood, Bere Regis, Dorset), plate counts (thousands of viable units/g.* D.W.), *and nature and abundance of species isolated*

Vegetation. Good stand of old Scots Pine, with sparse *Pteridium* and *Rubus* ground cover. Profile structure: A_0 (0–3 in.), surface litter and humus; A_1 (3–5 in.), sand containing humus, with some clay; A_2 (5–10 in.), sand with clay content increasing with depth; B (10–13 in.) iron pan; C (13–26 in.), grey clay with little sand.

Occurrence of species: + + + = abundant; + + = frequent; + = rare; . = not observed. D.W. = dry weight, F.W. = fresh weight of soil.

Sample no.	Depth (in.)	Horizon	pH	D.W./F.W. (%	Plate count	*A. glauca*	*Absidia orchidis*	*Aspergillus* sp. 912	*Cephalosporium acremonium*	*Cladosporium herbarum*	*Gliomastix convoluta*	*M. alpina*	*Mortierella isabellina*	*Mucor ramannianus*	*Papularia sphaerosperma*	*P. citreoviride*	*P. citrinum*	*P. frequentans* f. B.	*P. godlewskii*	*P. melinii*	*P. nigricans* f. A	*P. paxilli*	*P. roqueforti*	*P. stoloniferum*	*P. thomii*	*Penicillium* sp. 895	*Phoma* sp. 802	*Pilaira anomala*	*Pullularia pullulans*	*Sporotrichum* sp. 1038	*Torula* sp. 787	*Trichocladium asperum*	*Trichoderma viride*	'Fungus B'	Total no. of species
1	0–1	A_0	4·6	43	615	+++	.	.	.	.	.	+	.	++	.	.	+	.	++	++	+++	.	+++	.	.	.	.	.	+++	.	.	.	+++	+	11
2	1–3	A_0	3·9	58	1410	.	.	+++	.	.	.	.	.	.	+	.	++	++	+	++	+++	.	.	.	.	+	.	+	.	.	+	.	+++	+	12
3	3–5	A_1	3·8	83	60	+	+	+++	+++	.	.	.	.	+	.	+	.	.	.	+	+	.	.	.	.	.	.	.	.	+	.	+	.	.	11
4	5–10	A_2	4·1	91	35	+	.	+++	.	.	.	.	+	+++	.	.	+	.	.	+	+++	.	.	.	+	+	+	.	.	.	++	.	+++	.	12
5	10–12	B	4·4	91	14	.	.	++	.	.	+	.	+	+++	.	.	++	.	.	.	+++	.	.	.	.	++	.	.	.	.	+	.	+++	.	9
6	12–13	B	4·6	93	3	.	.	.	.	+	+	.	.	+++	.	.	+	.	.	.	++	+	.	++	.	+	.	.	.	.	++	.	+	.	10
7	13–18	C	5·1	92	<1	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
8	18–23	C	4·3	85	<1	.	.	.	.	+	.	.	.	.	.	+	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	3
9	23–26	C	4·0	86	<1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0

Table 9. *Characteristics of profile VIII (Sugar Hill, Bloxworth Heath, Wareham, Dorset), plate counts (thousands of viable units/g.* D.W.*), and nature and abundance of species isolated*

Vegetation. Felled Pine plantation of poor growth, deep ground cover of *Deschampsia*, *Ulex* and *Calluna*. Profile structure: A_0 (0–1 in.), humus; A_1 (1–4 in.), sand containing humus; A_2 (4–6 in.), leached sand; B (6–9 in.), iron pan; C (9–19 in.), brown sand.

Occurrence of species. + + + = abundant; + + = frequent; + = rare; . = not observed. D.W. = dry weight; F.W. = fresh weight of soil.

Sample no.	Depth (in.)	Horizon	pH	D.W./F.W. (%)	Plate count	*Absidia orchidis*	*Cladosporium herbarum*	*Gliocladium* sp. 650	*M. alpina*	*Mortierella isabellina*	*Mucor ramannianus*	*Oidiodendron griseum*	*P. lividum*	*P. melinii*	*P. nigricans* f. A	*P. nigricans* f. B	*P. roqueforti*	*P. rubrum*	*P. terlikowskii*	*P. thomii*	*Penicillium* sp. 895	*Sporotrichum* sp. 1038	*Trichoderma viride*	*Verticillium* sp. 1027	'Fungus A'	'Fungus B'	Total no. of species
1	0–1	A_0	4·5	63	306	+	.	.	.	+	+	.	++	+	+++	.	.	+	++	+	.	.	+++	.	.	.	10
2	1–4	A_1	4·5	69	96	.	.	.	.	.	.	.	.	.	++	+++	.	.	+	.	.	.	+++	.	.	.	4
3	4–6	A_2	4·4	77	210	.	.	.	.	.	.	.	.	++	+++	+++	.	.	+	.	.	.	++	+	+++	.	7
4	6–9	B	4·2	87	48	.	.	.	+++	.	.	.	.	.	+++	.	+++	.	+++	.	.	.	++	.	++	.	6
5	9–13	C	4·1	79	2	.	.	+	+	.	+	.	.	.	+	+	.	.	.	.	.	.	++	.	.	.	6
6	13–16	C	4·4	89	2	.	+	.	+	.	.	+	.	+++	+++	.	.	.	.	++	+++	.	++	.	.	+	9
7	16–19	C	4·6	91	2	.	+	.	.	.	.	.	.	.	+++	.	+	.	.	.	+	+	++	+	.	.	7

Table 10. *Characteristics of profile IX (Stroud Bridge, Wareham, Dorset), plate counts (thousands of viable units/g.* D.W.), *and nature and abundance of species isolated*

Vegetation. Recently fired boggy ground with little remaining surface vegetation other than a little regenerating *Calluna*; *Drosera* locally abundant. Profile structure: A_0 (0–1 in.), humus; A_1 (1–3 in.), silty sand containing humus; A_2 (3–15 in.), leached sand with a high proportion of fine silt, grading imperceptibly into C (15–20 in.), silty sand. Water level at time of sampling, 15 in.

Occurrence of species. + + + = abundant; + + = frequent; + = rare; . = not observed. D.W. = dry weight, F.W. = fresh weight of soil.

Sample no.	Depth (in.)	Horizon	pH	D.W./F.W. (%)	Plate count	*Cladosporium herbarum*	*Coniothyrium* sp. 977	*M. alpina*	*Mortierella isabellina*	*Mucor ramannianus*	*P. adametzi*	*P. frequentans* f. B	*P. melinii*	*P. nigricans* f. A	*P. roqueforti*	*P. stoloniferum*	*P. terlikowskii*	*P. thomii*	*Penicillium* sp. 895	*Pestalotia* sp. 799	*Phoma* sp. 1015	*Sporotrichum* sp. 1038	*Sclerotium* sp. 1077	*Trichoderma viride*	Total no. of species
1	0–1	A_0	4·3	49	44	++	.	+++	++	+	++	++	.	+	++	.	+	.	++	+	.	+	.	+++	13
2	1–3	A_1	4·1	81	5	.	+++	.	.	.	.	.	.	.	.	.	.	+	.	.	+	+	+++	.	5
3	3–6	A_2	4·3	84	2	.	.	.	+	.	.	.	+	.	++	+	.	.	.	.	.	.	.	.	4
4	6–10	A_2	4·5	84	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	1
5	10–15	A_2	4·4	86	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+++	.	.	1
6	15–20	C	4·7	82	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0

counts in all but the uppermost samples are probably a result of waterlogging.

The distribution of species is discussed in a later section of this paper.

Antibiotic production

As explained earlier, all representative cultures were tested for antibiotic production by an agar streak test and selected cultures were tested in liquid media. After each species listed below, two numbers are given; the first is the number of cultures tested in the agar streak test, the second is the number tested in liquid media. For convenience, species which have shown consistent indication of antibiotic production by the tests described are called 'active' species, those which have not are called 'inactive'.

PHYCOMYCETES

The following species were inactive in both tests:

Absidia orchidis (12; 2), *Mucor racemosus* (1; 1), *M. varians* (1; 1), *Pilaira anomala* (1; 1), *Zygorhyncus moelleri* (8; 2). Some isolates of the following species showed signs of antibacterial activity in the streak test, not unequivocally confirmed in liquid media: *Mortierella alpina* (19; 1), *M. isabellina* (7; 4), *M. vinacea* (1; 1). They showed no antifungal activity.

Two species showed more definite activity:

Absidia glauca (3; 2). Although no activity was seen in agar streak tests, both cultures tested on liquid media produced culture filtrates slightly but definitely inhibitory to *Bacillus subtilis*.

Mucor ramannianus (47; 8). Only one isolate was inactive in the streak test; all the remainder inhibited *Staph. aureus* and thirty-five inhibited *Salmonella typhi*. In liquid media, seven isolates produced culture filtrates inhibitory to *Bacillus subtilis*.

It is worthy of note that no antifungal activity has been shown by either of these species. This suggested that the inhibition of bacteria might have been a simple effect of pH, but the observed data do not support this view. No antibiotic has yet been isolated.

ASCOMYCETES

Sordaria fimicola (2; 2) was inactive in both tests.

FUNGI IMPERFECTI

Sphaeropsidales. *Phoma* spp. 802 (5; 2), 1015 (2; 1) and 1079 (1; 1) were inactive or nearly so in both tests.

Coniothyrium sp. 977 (3; 3). All isolates were generally antibacterial in the streak test. Suitable liquid media for adequate growth have not yet been found, but in such experiments as have been done the culture filtrates were slightly but definitely inhibitory to *Bacillus subtilis* and caused a rather unusual distortion of germ-tubes of *Botrytis*.

Melanconiales. *Pestalotia* sp. 799 (1; 1) was inactive in both tests.

Moniliales. The following species were inactive in both tests:

Aspergillus ruber (2; 1), *Cephalosporium acremonium* (3; 2), *Cladosporium herbarum* (53; 7), *Gliomastix convoluta* (2; 2), *Penicillium charlesii* (1; 1), *P. citreoviride* (6; 2), *P. fellutanum* (3; 2), *P. lanosum* (1; 1), *P. roqueforti* (22; 4), *P. thomii* (7; 3), *Penicillium* sp. 895 (22; 4), *Scopulariopsis* sp. 1005 (1; 1), *Scopulariopsis* sp. 1068 (2; 1), *Sporotrichum* sp. 1038 (12; 3), *Torula* sp. 787 (12; 3), *Torula* sp. 781 (1; 1), *Trichocladium asperum* (1; 1).

Active species are dealt with in turn below:

Aspergillus sp. 912 (7; 2). This appears to be a previously undescribed species. In the agar streak test two strains were inactive. All the remaining five showed some antibacterial activity, two of them being markedly inhibitory to all the test bacteria and to *Candida albicans*. Both strains tested in liquid media slightly but definitely inhibited *Botrytis* and inhibited *B. subtilis* and *Bact. coli* markedly.

Gliocladium sp. 650 (*roseum* series) (9; 5). In the streak test four isolated were inhibitory to *Staph. aureus*. Five isolates, including some of those inactive in the streak test, all produced culture filtrates in liquid media inhibiting *Botrytis* and *B. subtilis*. From one of these the related antibiotics gliorosein, rubrogliocladin and aurantiogliocladin have been obtained (Brian, Curtis, Howland, Jefferys & Raudnitz, 1951; Vischer, 1953).

Oidiodendron griseum (1; 1) gave marked antibacterial activity in liquid media, though not in the streak test.

Papularia sphaerosperma (1; 1) was inactive in the agar streak test but in liquid media slightly but definitely inhibited *Botrytis* and *B. subtilis*.

Penicillium adametzi (11; 2). Nearly all strains were quite inactive in the streak test; two showed traces of antifungal or antibacterial activity. In liquid media both strains tested produced culture filtrates slightly toxic to *Botrytis*.

P. albidum (9; 5). All nine isolates inhibited all the test bacteria in the agar streak test and all but two also inhibited *Candida albicans*. Five isolates grown in liquid media produced antifungal and antibacterial culture filtrates. The antibiotic albidin has been obtained from an earlier isolation of this species (Curtis & Grove, 1947; Curtis, Hemming & Unwin, 1951) and also from one of the isolates made in this study (Curtis, unpublished results).

P. chrysogenum (1; 1). This isolate inhibited *Staph. aureus* in the agar streak test and in liquid media produced culture filtrates inhibitory to *B. subtilis* and slightly but definitely inhibitory to *Botrytis*. The antibacterial activity is probably due to penicillin but the nature of the antifungal substance is unknown.

P. citrinum (4; 3). These strains differed from typical *P. citrinum* in that the chains of spores were not strictly columnar; they undoubtedly fall into the *P. citrinum* series and since they produce yellow colours in reverse on Czapek agar it seems preferable to group them as *P. citrinum* rather than *P. steckii*, the only other possibility. All were inhibitory to *Staph. aureus* in the streak test, two strains also inhibited *Salmonella typhi*. In liquid media they produced yellow, antibacterial and slightly antifungal culture filtrates.

On adding ferric chloride, a thick brown precipitate was formed, dissolving in excess ferric chloride. This strongly suggests production of the antibiotic citrinin, a well-known metabolic product of this species.

P. frequentans. Two forms of this species were isolated, differing in colonial appearance and antibiotic production. One of these (form A) was typical of the species as described by Raper & Thom (1949); colonies on Czapek agar were broadly spreading, dark green and with abundant yellow-brown pigment in reverse. The other (form B) was a light greyish-green colour with the reverse uncoloured or pale yellow.

Form A (3; 0). Two strains were inactive in the streak test and two showed slight general antibacterial activity. From strains of similar appearance we have obtained citromycetin, an antibiotic of low activity (Grove & Brian, 1951).

Form B (29; 12). In the streak test three isolates inhibited all the test organisms, ten inhibited all the bacteria but not *Candida*, and three inhibited at least one of the test bacteria. In liquid media ten of the twelve strains tested produced culture filtrates inhibiting *Botrytis* strongly and *B. subtilis* moderately. The antibiotic frequentin has been isolated (Curtis, Hemming & Smith, 1951).

P. godlewskii (3; 3). Two isolates were antibacterial in the streak test and one was inactive. All produced highly antifungal and antibacterial culture filtrates in liquid media. No antibiotic has yet been isolated.

P. lividum (4; 1). Three isolates were inactive in the streak test but one was inhibitory to all the organisms. This isolate produced slightly antibacterial and antifungal culture filtrates in liquid media.

P. melinii (14; 6). Six isolates showed general antibacterial and slight antifungal activity; the remainder were inactive. Of the six strains tested in liquid media, four were inactive. The remaining two strains produced highly antifungal culture filtrates with some activity against *B. subtilis*. No antibiotic has yet been isolated.

P. nigricans. Two forms of this species were isolated differing in colonial appearance and antibiotic activity. One produced deeply floccose colonies, sporulating well on Czapek agar and with a pronounced and characteristic mouldy smell; this (form A) was the same as that we have previously worked with under the name *P. janczewskii* Zal. Form B was more broadly spreading, much thinner and less floccose, sporulation on Czapek agar was scanty and the odour was less pronounced. Form A was much more common than form B.

Form A (64; 5). Fifty-three isolates inhibited only *Candida* in the streak test; three strains inhibited one or more of the bacteria in addition; the remainder were inactive. All of the five strains tested produced culture filtrates in liquid media which produced distortion of *Botrytis* germ-tubes characteristic of griseofulvin; from some of these strains griseofulvin and dechlorogriseofulvin have been isolated (Brian, Curtis & Hemming, 1946; Grove & McGowan, 1947; Brian, 1949*c*; Brian, Curtis & Hemming, 1949; MacMillan, 1951; Grove, MacMillan, Mulholland & Rogers, 1952).

Form B (13; 3). Eleven strains showed activity against all test organisms. In liquid media culture filtrates moderately active against *Botrytis* and *B. subtilis* were produced. It seems likely that this activity is due to production of albidin, previously shown to be produced by the closely related *P. albidum.*

P. notatum (2; 1). Though both isolates were inactive in the streak test, one was grown in liquid media and produced culture filtrates inhibitory to *B. subtilis.* This activity was probably due to penicillin, but the amount formed was certainly small.

P. palitans (3; 1). One isolate inhibited *Candida* in the streak test, the others were inactive. The active strain produced highly antifungal culture filtrates in liquid media. The antibiotic frequentin (also produced by *P. frequentans*) has been isolated together with the chemically related substance palitantin which, however, is antibiotically inactive (Birkinshaw, 1952; Curtis & Duncanson, 1952).

P. paxilli (2; 1). Both isolates showed antibacterial and antifungal activity in the streak test. One was tested in liquid media and was found to produce culture filtrates with marked 'germ-tube stunting' activity against *Botrytis* characteristic of that produced by mycophenolic acid. From another isolate of this species, which showed similar activity, mycophenolic acid was isolated (Brian, 1951).

P. rubrum (1; 1) caused inhibition of *Salmonella typhi* in the streak test and produced culture filtrates in liquid media slightly inhibitory to *Botrytis* and *B. subtilis.*

P. spinulosum (16; 5). All isolates were inactive in the streak test, but four produced, in liquid media, culture filtrates moderately inhibitory to *Botrytis* and *B. subtilis.*

P. stoloniferum (25; 3). *Candida* was inhibited in the streak test by twenty-one isolates, the remainder being inactive. The three isolates grown in liquid media produced culture filtrates slightly inhibitory to *B. subtilis* and causing marked stunting of germ-tubes of *Botrytis.* On the basis of previous experience with this species we can with some confidence attribute the activity to mycophenolic acid and related substances.

P. tardum (2; 1). Both strains were generally antibacterial in the streak test. One was tested in liquid media, and produced slightly antibacterial culture filtrates.

P. terlikowskii (21; 4). Most of these strains agreed perfectly with the description of the species given by Raper & Thom (1949), but six produced abundant small sclerotia, being otherwise similar. In correspondence, Dr K. B. Raper has agreed that these are the same species and that the description of the species should be expanded to cover occasional production of sclerotia. Of the twenty-one isolates tested on agar, ten were completely inactive, three inhibited all the test organisms, and the remainder inhibited *Candida* alone or *Candida* and *Staph. aureus.* Four were selected for test in liquid media, including ones active and inactive on agar, and sclerotial and non-sclerotial strains. All produced culture filtrates highly inhibitory to

Botrytis, *B. subtilis* and *Bact. coli*. This has been shown to be due to production of gliotoxin by both non-sclerotial (Brian, 1946) and sclerotial strains (Curtis, unpublished results).

P. urticae (5; 1). One strain was inactive in the agar streak test, one inhibited all test organisms and the remainder inhibited at least two of the test organisms. One isolate was grown in liquid media and the bioassay results indicated the production of griseofulvin and some other antibiotic toxic to both fungi and bacteria; the latter is probably patulin which was obtained from this species (under the name *P. patulum*) by Anslow, Raistrick & Smith (1943).

Penicillium sp. 1006 (*raistrickii* series) (1; 1). Though inactive in the streak test, in liquid media this fungus produced culture filtrates which caused a marked stunting of *Botrytis* germ-tubes. This organism differs from *P. raistrickii* Smith in having smooth conidiophores and tangled conidial chains; otherwise it is very similar.

Pullularia pullulans (3; 1). All isolates were generally antibacterial in the streak test. The one strain grown in liquid culture media produced culture filtrates inhibitory to *B. subtilis* and slightly inhibitory to *Botrytis*.

Scopulariopsis sp. 809 (1; 1) showed marked inhibition of *Staph. aureus* in the streak test but in liquid media only slight activity against *B. subtilis* was recorded.

Trichoderma viride (140; 15). This species was isolated very frequently. There was considerable variation in colonial characters and all intermediates between 'pustulate' and 'floccose' types were found, but colonial appearance and antibiotic production were not correlated. Of the 140 isolates tested on agar, forty-five were inactive, thirty-nine inhibited all test organisms, thirty inhibited all test bacteria, fourteen inhibited *Candida* only and the remaining twelve inhibited one or more of the bacteria. Fifteen strains, representing all types of activity in the streak test, were grown in liquid media; four were inactive, two inhibited *B. subtilis* only and nine inhibited both *Botrytis* and *B. subtilis*. The impression gained from bioassay, based on previous experience with this species, was that gliotoxin and viridin probably accounted for the activity. None produced culture filtrates as potent as those from strains from which we have previously isolated viridin (Brian, Curtis, Hemming & McGowan, 1946) and gliotoxin (Brian & Hemming, 1945).

Verticillium sp. 1027 (5; 3). All strains were inactive on agar media but in liquid media two produced appreciable antifungal and antibacterial activity.

Mycelia sterilia. *Sclerotium* sp. 1077 (2; 2), 'fungus A' (29; 1) and 'fungus B' (11; 1) were inactive in both types of test.

Tolerance of antibiotics by soil fungi

The effect on germination of spores of some of the more common fungi from these soils to antibiotics produced by fungi also isolated from these soils, is shown in Table 11. The antibiotics (aurantio-gliocladin, albidin, citrinin, frequentin, griseofulvin, gliotoxin, patulin, and viridin) were made up at 50 μg./ml. in a germination medium (Brian & Hemming, 1945) of pH 4·0 and

Table 11. *Effect of antibiotics on germination of spores of soil fungi at* pH 4·0

(A mark of 0 indicates no inhibition of germination at 50 μg./ml.; 1 indicates inhibition at 50 μg./ml., but not at 25 μg./ml., and so on.)

	Aurantio-gliocladin	Albidin	Citrinin	Frequentin	Griseo-fulvin	Gliotoxin	Patulin	Viridin	Total value for all antibiotics
Group 1. Saprophytes which do not produce antifungal antibiotics:									
Absidia orchidis	0	4	4	1	1	2	0	5	17
A. glauca	0	3	3	0	0	1	0	8	15
Cephalosporium acremonium	4	5	3	3	1	4	1	11	32
Mortierella isabellina	2	6	4	1	2	5	0	7	27
Mucor racemosus	0	6	3	4	0	1	0	6	20
M. ramannianus	0	6	4	2	1	5	0	5	23
Penicillium roqueforti	0	2	3	0	2	2	0	9	18
Sporotrichum sp. 1038	4	7	3	3	4	0	0	10	31
Zygorhyncus moelleri	0	2	3	0	2	2	0	9	18
Mean	1·1	4·6	3·3	1·6	1·4	2·4	0·1	7·8	22·3
Group 2. Saprophytes which produce antifungal antibiotics:									
Aspergillus sp. 912	0	3	3	2	5	2	0	10	25
Gliocladium sp. 650	**0**	4	1	2	4	1	0	7	19
Penicillium albidum	2	**1**	2	3	1	3	0	10	22
P. citrinum	3	5	**0**	3	1	2	0	10	24
P. frequentans f. B	2	3	0	**0**	5	1	1	8	20
P. nigricans f. A	1	3	3	3	**0**	0	0	8	18
P. terlikowskii	3	4	4	3	5	**0**	0	10	29
P. urticae	0	5	3	2	**0**	1	**0**	9	20
Trichoderma viride	0	2	1	2	7	2	0	**4**	18
Mean	1·2	3·3	1·9	2·2	3·1	1·3	0·1	8·4	21·7
Group 3. Plant parasites:									
Botrytis allii	2	7	5	5	9	4	0	11	44
Fusarium caeruleum	1	5	4	6	4	4	1	11	35
F. graminearum	1	6	2	3	5	4	0	10	31
Helminthosporium sativum	0	4	3	1	7	1	0	10	26
Mean	1·0	5·5	3·5	3·8	6·3	3·3	0·3	10·5	34·0

(*Note.* Values in bold type indicate the effect of an antibiotic on a fungus which itself produces it.)

spore germination tests were carried out in this medium and in a series of 1/2 dilutions. In Table 11, a value of 0 indicates less than 95 % inhibition of spore germination at 50 μg./ml., 1 indicates more than 95 % inhibition at 50 μg./ml. but not at 25 μg./ml. and so on. In the case of griseofulvin the criterion used was not inhibition of germination, but production of a recognizable morphogenetic response in the germ-tube. A number of plant-parasitic fungi, for the most part usually soil-borne, were included for purposes of comparison.

It will be seen first that the plant parasites (group 3) were on the whole the most susceptible, as has already been noted by Garrett (1950). In this group *Helminthosporium sativum* was anomalous in being distinctly resistant; it is perhaps significant that this species itself produces an antifungal antibiotic whereas the other species in the group do not (Brian & Hemming, unpublished results). Secondly, a fungus which itself produces an antifungal antibiotic is in all cases markedly resistant to that antibiotic, though not necessarily to others. Thirdly, those fungi which, though of common occurrence, do not produce antifungal antibiotics (group 1), are not in general more susceptible than the antibiotic producers (group 2). Indeed, the two most generally resistant fungi, *Absidia orchidis* and *A. glauca*, do not produce antifungal antibiotics.

Utilization of carboxymethylcellulose

Representative cultures of all species isolated were tested for their capacity to break down sodium carboxymethylcellulose (CMC). They were grouped as follows: (*a*) those unable to attack CMC (CMC −); (*b*) those able to attack CMC slowly, appreciable quantities being unchanged in 14 days (CMC +); (*c*) those attacking CMC vigorously, little if any being left after 14 days' growth of the fungus (CMC + +). Of these, category (*b*) is probably the least reliable. The results are embodied in Table 1.

In most cases all cultures of any one species behaved similarly. Some exceptions are worthy of note. Of the seven cultures of *Trichoderma viride* tested, one was completely unable to utilize CMC. Of the six cultures of *Cladosporium herbarum*, one was completely unable to utilize CMC. Of the four cultures of *Gliocladium* sp. 650 tested, one attacked CMC vigorously, two attacked it slowly and one was completely unable to utilize CMC. Similar variation from vigorous attack to complete inability to attack was also seen with *Penicillium spinulosum* and *Verticillium* sp. 1027.

DISCUSSION

Distribution of species

The most widespread species, found in at least four of the nine profiles, were: *Absidia orchidis*, *Cladosporium herbarum*, *Mortierella alpina*, *M. isabellina*, *Mucor ramannianus*, *Penicillium albidum*, *P. frequentans* f. B, *P. roqueforti*, *P. spinulosum*, *P. stoloniferum*, *P. terlikowskii*, *P. thomii*, *Penicillium* sp. 895, *Sporotrichum* sp. 1038, *Trichoderma viride*, 'fungus A' and 'fungus B'. Of these, *T. viride* was the most commonly found. Nearly all these widely distributed species were found abundantly in at least one soil sample.

The following additional species were also found abundantly in at least one soil sample but were found in less than four profiles, i.e. were locally abundant: *Absidia glauca*, *Aspergillus* sp. 912, *Cephalosporium acremonium*, *Coniothyrium* sp. 977, *Gliocladium* sp. 650, *Penicillium adametzi*, *P. citrinum*, *Pullularia pullulans*, *Sclerotium* sp. 1077, *Scopulariopsis* sp. 1005 and *Zygorhyncus moelleri*.

Of the widespread species, nearly all were more abundant in surface layers. Some, such as *Absidia orchidis* and *Penicillium terlikowskii*, were typically found in the A_0 and A_1 horizons, occurring only sporadically at lower levels. Others, such as *Cladosporium herbarum*, *Mortierella alpina*, *Penicillium nigricans*, *P. roqueforti*, *Penicillium* sp. 895 and *Trichoderma viride*, though usually found in surface layers, were also not infrequently found in A_2, B and C horizons. *Mucor remannianus*, on the other hand, was most commonly found in the lower levels of the A horizon and in the B horizon, even occurring abundantly in the C horizon in some profiles. It appears to be better adapted for existence at these lower levels, and, in fact, becomes the dominant fungus in such situations. In some samples (samples 5 and 6 of profile I, samples 5, 6 and 7 of profile IV and sample 7 of profile VII) it was present in almost pure culture. Another fungus which seems to show a similar adaptation for life in lower levels of soil is *Sporotrichum* sp. 1038. It was present in pure culture in lower levels of the waterlogged profile IX.

The distribution of *Mucor ramannianus* among the various profiles is interesting. It was found as an abundant and characteristic feature in six of the nine profiles. In three profiles it was completely absent. This may be related to the occurrence of a well-marked iron pan in the B horizon, for in all such profiles *M. ramannianus* was present and usually particularly abundant in the pan layer; in those with an ill-defined B horizon it was absent. This may have been purely fortuitous but is worthy of record.

There is an obvious likeness in the mould flora of the nine profiles studied and such species as *Absidia orchidis*, *Mortierella alpina*, *Mucor ramannianus*, *Penicillium nigricans*, *P. terlikowskii* and *Trichoderma viride* appear to be characteristic of these acid sandy soils. This is very similar to the mould flora found by Warcup (1951) in an acid sandy podsol (grassland E) on Lakenheath Warren, Suffolk, with two exceptions worthy of note. He found *Zygorhyncus vuilleminii* to be of regular occurrence. We have not found this species, though in one profile (III) we recorded what we regard as *Z. moelleri*, on the basis of comparison with authentic cultures from the Centraalbureau voor Schimmelcultures, Baarn, Holland. The fungus was abundant in the A horizon but absent from lower layers. This distribution is similar to that of *Z. vuilleminii* described by Warcup, in distinction from that described by Waksman (1917), who found it characteristically in the subsoil. Secondly, though Warcup found *Mortierella alpina* in neutral and alkaline soils, he did not find it in acid soils. He suggested, and quoted supporting data from Linnemann (1941), that it is normally found in soils of pH 6·0–8·6. As recorded above, we have found *M. alpina* in soils of pH 3·9–4·6, though less abundantly than in certain alkaline cave soils not dealt with here. Linnemann

(1941) mentions finding it once in an acid forest soil of pH 3·7. It is remarkable that this species was found in all the Wareham profiles but in none of those from Bagshot Heath. *M. isabellina* also was not found in the Bagshot Heath soils though found in four of the five profiles from Wareham. It may be that the time of sampling (January for Wareham, October for Bagshot) was the decisive factor. Warcup (1951) has recorded a species of *Pythium* which could be isolated readily in the winter months but not at other times of the year.

Antibiotic production in relation to distribution of soil fungi

The results of experiments in liquid media probably afford a better criterion than agar streak tests of the capacity of a fungus to produce antibiotics. That criterion is therefore used exclusively in the following discussion. Of the sixty-five species isolated in this investigation, about half produce antibiotics (Table 12). Most frequently, culture filtrates (and the pure antibiotics, where isolated) are inhibitory to both bacteria and fungi. Selective inhibition of one or the other is much less common. This preponderance of general toxicity is characteristic of the fungal antibiotics.

Table 12. *Analysis of numbers of species of fungi from acid sandy soils producing culture filtrates on liquid media inhibitory to* (*a*) *fungi alone;* (*b*) *bacteria alone;* (*c*) *fungi and bacteria*

Group	No. of species tested	Inactive	Active		
			(*a*)	(*b*)	(*c*)
Phycomycetes	10	5	0	5	0
Ascomycetes	1	1	0	0	0
{Penicillia	28	7	4	1	16
{Other Fungi Imperfecti	26	18	0	2	6
Total	65	31	4	8	22

There is some evidence of a correlation between the capacity to produce antibiotics, especially antibiotics with antifungal properties, and the frequency of occurrence of species. This can be seen by comparing those species which we have described above as widespread species (excluding *Cladosporium herbarum* and *Penicillium stoloniferum* because of their doubtful status as soil inhabitants), those species which we have described as locally abundant species, and a group of rare species (*Aspergillus ruber, Gliomastix convoluta, Mortierella vinacea, Mucor racemosus, M. varians, Penicillium charlesii, P. chrysogenum, P. fellutanum, P. lanosum, P. palitans, P. paxilli, P. rubrum, P. tardum, Pestalotia* sp. 799, *Phoma* spp. 802 and 1015, *Pilaira anomala, Scopulariopsis* spp. 809 and 1068, *Torula* sp. 781 and *Trichocladium asperum*). These rare species are all those found in not more than two samples and graded as rare in those. The percentage of species, in each of these groups, producing antifungal antibiotics are:

Widespread fungi (18 spp., 86 cultures tested)	45 %
Locally abundant fungi (11 spp., 25 cultures tested)	45 %
Rare fungi (21 spp., 24 cultures tested)	15 %

Thus there appears to be a distinct tendency for abundance to be associated with the capacity to produce antifungal substances. It could be argued that in arriving at these figures a bias was introduced in favour of the more common species, since a species has been considered to be active even if some strains were inactive and since in general more cultures of the commoner species were examined. If a correction is made for this bias by proportionately reducing the marking for any species where some cultures were active but others inactive, the percentage of species producing antifungal substances only changes slightly, becoming 35, 45 and 15 % respectively.

If the capacity to produce all antibiotics is considered, i.e. including also those specifically antibacterial, the contrast between these groups is somewhat less marked, becoming (for corrected values) 49, 64 and 30 % respectively.

It might be considered that this apparent tendency for antibiotic-producing fungi to be more abundant is really a reflexion of antagonism in the isolation plates, those producing antifungal antibiotics tending to inhibit the development of others. Care was taken to avoid overcrowding on the isolation plates and such an explanation seems unlikely.

At the same time it should be noted that quite a number of widespread fungi do not produce antifungal antibiotics, viz. *Absidia orchidis*, *Mortierella alpina*, *M. isabellina*, *Mucor ramannianus*, *Penicillium roqueforti*, *P. thomii*, *Penicillium* sp. 895, *Sporotrichum* sp. 1038, and 'fungi A and B'. Among the locally abundant fungi, the following species do not produce antifungal antibiotics: *Absidia glauca*, *Cephalosporium acremonium*, *Pullularia pullulans*, *Sclerotium* sp. 1077, *Scopulariopsis* sp. 1005 and *Zygorhyncus moelleri*. As we have seen (Table 11), such of these species as have been examined are, as a class, resistant to the antibiotics produced by other fungi isolated from these soils, more so, for example, than a group of plant-parasitic fungi. Of course, this resistance could also be taken to explain their vigorous growth on isolation plates in the presence of antibiotic-producing fungi, but, as explained above, this interpretation is not considered adequate.

Sugar fungi and humus fungi

Burges (1939) has distinguished two ecological groups among the saprophytic soil fungi. Some can develop the necessary enzyme systems to degrade and utilize such relatively resistant plant materials as cellulose and other cell-wall constituents; they can consequently become primary invaders of such dead plant materials in the soil. Others cannot degrade such material and need much simpler carbon compounds, such as sugars, for growth; these are consequently to a great extent dependent on the activities of the former group and are only secondarily invaders of dead plant material. Burges calls these two groups humus-fungi and sugar-fungi respectively.

Using carboxymethylcellulose (CMC) as a convenient model substrate, similar to cellulose in its resistance to fungal attack (Siu, 1951), we roughly divide our species into sugar-fungi and humus-fungi. The capacity to attack CMC *in vitro* does not necessarily indicate a capacity to attack resistant plant residues in the soil; indeed Siu (1951) has recorded some cases of fungi capable of attacking cellulose or CMC *in vitro* which apparently cannot or do not attack cellulose in the soil. There is likely to be some correlation, nevertheless, between *in vitro* activity and activity in soil. Furthermore, those fungi which cannot attack CMC *in vitro* almost certainly will not be able to attack cellulose or other cell-wall materials in the soil and so can fairly be considered to be sugar-fungi in the sense used by Burges.

The proportions of sugar-fungi, determined in this way, in various categories of the fungi isolated are given in Table 13. These data serve to confirm the validity and importance of Burges' ecological grouping.

Table 13. *Proportion (%) of sugar-fungi (as determined by failure to degrade carboxymethylcellulose) in various categories of fungi isolated from acid sandy podsols*

	All species	Phycomycetes excluded
All fungi	26	20
Phycomycetes	70	—
Widespread fungi	17	0
Locally abundant fungi	54	45
Rare fungi	33	35
All antibiotic-producers	34	18
Antifungal antibiotic-producers	17	17
Inactive species	27	24

The most striking feature of these results is that whereas only 26 % of all fungi isolated can be characterized as sugar-fungi, no less than 70 % of the Phycomycetes are sugar-fungi. This characteristic of the Phycomycetes is also to be seen in the data compiled by Siu (1951).

Equally striking is the very low proportion of sugar-fungi in the widespread species listed in the earlier section; apart from the Phycomycetes which fall in this category, none of the widespread fungi are sugar-fungi. It thus appears that the capacity to degrade resistant plant-materials is closely correlated with wide distribution, though certain Phycomycetes (e.g. *Absidia orchidis*, *Mortierella alpina*, *M. isabellina*) achieve wide distribution without that capacity.

A third noteworthy feature is that rather fewer of the fungi which produce antibiotics toxic to other fungi are sugar-fungi. This is not the case with antibiotically inactive fungi, nor with all antibiotic-producers considered together. This is largely due to a bias introduced by the Phycomycetes, which as a group combine inactivity in CMC-degradation with inability to produce antibiotics toxic to other fungi. If the Phycomycetes are ignored, there is little difference in the proportion of sugar-fungi in the various categories based on antibiotic production.

CONCLUSION

The results of this survey support the thesis that antibiotic production by soil fungi is of ecological significance. But this support is not unequivocal and is in any case indirect and circumstantial. More direct evidence is required. Organisms from this type of soil which would probably repay further investigation include the following species: *Penicillium albidum, P. nigricans* f. A, *P. terlikowskii, P. frequentans* f. B, *P. citrinum, Trichoderma viride* and *Mucor ramannianus*. A good deal is known of the antibiotics produced by some of these species, and the following are of greatest potential importance in this type of soil: albidin, griseofulvin, dechlorogriseofulvin, gliotoxin, viridin, frequentin and citrinin. We have not yet isolated an antibiotic from *Mucor ramannianus*; this is a matter of some interest, since no one has as yet recorded the isolation of an antibiotic from a phycomycete.

We are indebted to Mr E. W. Mason, Dr K. B. Raper and Mrs Myfanwy Turner for help in the identification of some cultures; to Dr J. H. Warcup for giving us cultures of fungi isolated from Breckland soils; to Mr P. J. Curtis for allowing us to quote some unpublished results on antibiotics produced by these fungi; to Miss Gwenda Batt, Miss Olive McMinn and Mr Ronald Weaver for technical assistance.

REFERENCES

ANSLOW, W. K., RAISTRICK, H. & SMITH, G. (1943). Antifungal substances from moulds. Part I. Patulin, a metabolic product of *Penicillium patulum* Bainier and *P. expansum* (Link) Thom. *Chem. Ind., Lond.* p. 236.

BIRKINSHAW, J. H. (1952). Studies in the biochemistry of micro-organisms. Palitantin. Part 2. Further derivatives and degradation products. *Biochem. J.* **51** (2), 271–5.

BRIAN, P. W. (1946). Production of gliotoxin by *Penicillium terlikowskii* Zal. *Trans. Brit. mycol. Soc.* **29**, 211.

BRIAN, P. W. (1949*a*). The production of antibiotics by soil micro-organisms. *Chem. Ind., Lond.* p. 391.

BRIAN, P. W. (1949*b*). The production of antibiotics by micro-organisms in relation to biological equilibria in soil. *Symp. Soc. exp. Biol.* **3**, 357.

BRIAN, P. W. (1949*c*). Studies on the biological activity of griseofulvin. *Ann. Bot.* (N.S.), **13**, 59.

BRIAN, P. W. (1951). Antibiotics produced by fungi. *Bot. Rev.* **17**, 357.

BRIAN, P. W., CURTIS, P. J. & HEMMING, H. G. (1946). A substance causing abnormal development of fungal hyphae produced by *Penicillium janczewskii* Zal. I. Biological assay, production and isolation of 'curling factor'. *Trans. Brit. mycol. Soc.* **29**, 173.

BRIAN, P. W., CURTIS, P. J. & HEMMING, H. G. (1947). Glutinosin: a fungistatic metabolic product of the mould *Metarrhizium glutinosum* S. Pope. *Proc. Roy. Soc.* (B), **135**, 106.

BRIAN, P. W., CURTIS, P. J. & HEMMING, H. G. (1949). A substance causing abnormal development of fungal hyphae produced by *Penicillium janczewskii* Zal. III. Identity of 'curling-factor' with griseofulvin. *Trans. Brit. mycol. Soc.* **32**, 30.

BRIAN, P. W., CURTIS, P. J., HEMMING, H. G., JEFFERYS, E. G., UNWIN, C. H. & WRIGHT, J. M. (1951). Alternaric acid; a biologically active metabolic product of *Alternaria solani* (Ell. & Mart.) Jones & Grout; its production, isolation and antifungal properties. *J. gen. Microbiol.* **5**, 619.

BRIAN, P. W., CURTIS, P. J., HEMMING, H. G. & MCGOWAN, J. C. (1946). The production of viridin by pigment-forming strains of *Trichoderma viride*. *Ann. appl. Biol.* **33**, 190.

BRIAN, P. W., CURTIS, P. J., HOWLAND, S. R., JEFFERYS, E. G. & RAUDNITZ, H. (1951). Three new antibiotics from a species of *Gliocladium*. *Experientia*, **7**, 266.

Brian, P. W. & Hemming, H. G. (1945). Gliotoxin, a fungistatic metabolic product of *Trichoderma viride*. *Ann. appl. Biol.* **32**, 214.

Brian, P. W., Hemming, H. G. & McGowan, J. C. (1945). Origin of a toxicity to mycorrhiza in Wareham Heath soil. *Nature, Lond.* **155**, 637.

Burges, A. (1939). Soil fungi and root infection. *Broteria*, 8, 64.

Curtis, P. J. & Duncanson, L. A. (1952). A structural relationship between frequentin and palitantin. *Biochem. J.* **51**, 276.

Curtis, P. J., Hemming, H. G. & Smith, W. K. (1951). Frequentin: an antibiotic produced by some strains of *Penicillium frequentans* Westling. *Nature, Lond.* **167**, 557.

Curtis, P. J., Hemming, H. G. & Unwin, C. H. (1951). Albidin, an antibiotic red pigment from *Penicillium albidum*. *Trans. Brit. mycol. Soc.* **34**, 332.

Curtis, P. J. & Grove, J. F. (1947). A fungistatic and bacteriostatic red pigment produced by a strain of the *Penicillium nigricans-janczewskii* series. *Nature, Lond.* **160**, 574.

Garrett, S. D. (1950). Ecology of the root inhabiting fungi. *Biol. Rev.* **25**, 220.

Garrett, S. D. (1951). Ecological groups of soil fungi: a survey of substrate relationships. *New Phytol.* **50**, 149.

Gatenby, J. B. & Beams, H. W. (1950). *The Microtomist's Vade-mecum.* 11th ed. London: J. and A. Churchill Ltd.

Grove, J. F. & Brian, P. W. (1951). Identity of frequentic acid and citromycetin. *Nature, Lond.* **167**, 995.

Grove, J. F. & McGowan, J. C. (1947). Identity of griseofulvin and 'curling factor'. *Nature, Lond.* **160**, 574.

Grove, J. F., MacMillan, J., Mulholland, T. P. C. & Rogers, M. A. T. (1952). Griseofulvin. Part IV. Structure. *J. chem. Soc.* p. 3977.

Jefferys, E. G. (1952). The stability of antibiotics in soils. *J. gen. Microbiol.* **7**, 295.

Linnemann, G. (1941). *Die Mucorineen-Gattung* Mortierella *Coemans.* Jena: Gustav Fischer.

MacMillan, J. (1951). Dechlorogriseofulvin—a metabolic product of *Penicillium griseofulvum* Dierckx and *Penicillium janczewskii* Zal. *Chem. Ind., Lond.* p. 719.

Naumov, N. A. (1939). *Clés des Mucorinées.* Paris: Paul Lechevalier.

Neilson-Jones, W. (1941). Biological aspects of soil fertility. *J. agric. Sci.* **31**, 379.

Raper, K. B. & Thom, C. (1949). *A Manual of the Penicillia.* London: Baillière, Tindall and Cox.

Rayner, M. C. (1945). Origin of a toxicity to fungi in Wareham Heath soil. *Nature, Lond.* **156**, 174.

Siu, R. G. H. (1951). *Microbial Decomposition of Cellulose.* New York: Reinhold Publishing Corporation.

Thom, C. & Raper, K. B. (1945). *A Manual of the Aspergilli.* London: Baillière, Tindall and Cox.

Vischer, E. B. (1953). The structures of aurantio- and rubro-gliocladin and gliorosein. *J. chem. Soc.* p. 815.

Waksman, S. A. (1917). Is there any fungus flora of the soil? *Soil Sci.* **3**, 565.

Warcup, J. H. (1951). The ecology of soil fungi. *Trans. Brit. mycol. Soc.* **34**, 376.

Wright, J. M. (1952). Production of gliotoxin in unsterilized soil. *Nature, Lond.* **170**, 673.

Zycha, H. (1935). *Mucorineae.* (Bd. vi: *Kryptogamenflora der Mark Brandenburg.*) Leipzig: Gebrüder Borntraeger.

(*Received* 29 *April* 1953)

PARRY, W. R. & EDWARDS, J. (1953). *J. gen. Microbiol.* 9, 342–349.

The Induction of Lysogenesis in *Salmonella typhimurium*

By W. R. PARRY AND JOYCE EDWARDS

Microbiological Research Department, Porton, near Salisbury, Wiltshire

SUMMARY: After exposure to phage A1 an organism of an indicator strain of *Salmonella typhimurium* may burst with phage liberation, may become lysogenic, or may develop into a mixed progeny of lysogenic and phage-sensitive bacteria. By the methods we have used, the proportion of such mixed progenies is about 20% of the number of lysogenic colonies found. Evidence is given to show that members of the clone of phage A1 vary in virulence and that members of the indicator strain 1404 vary also in resistance to lysis. The establishment of lysogenesis depends on the interplay of these two factors when host and parasite meet.

The reaction of certain phages of *Salmonella typhimurium* with their host has been studied by Boyd (1951*b*). He regards a phage lysate as being a mixture of two kinds of particle: alpha particles which establish a state of lysogenesis (symbiosis) with an indicator organism, and beta particles which lyse the bacterial cell. Boyd postulates that the symbiotic state is the dominant one and therefore an organism exposed to an alpha particle (or to an alpha plus one or more beta particles) becomes lysogenic. While attempting to confirm these views, a type of colony was observed, infected with phage, and showing a central punctate nibbling. This phenomenon did not seem to arise as a result of the edge of a growing colony impinging on a free phage particle (Pl. 1, figs. 1 and 2). If this type of centrally nibbled colony could be shown to arise from a single cell the inception of lysogenesis in an indicator strain must be more complex than can be accounted for by Boyd's alpha and beta particle hypothesis. Lwoff & Gutmann (1950) who studied *Bacillus megaterium*, suggested that all the particles of phage in a lysate are alike and that the inception of a state of lysogenesis is due to a phage particle coming into contact with a resistant bacterium; the phage enters the bacterium and develops to the stage of prophage but no further. Neither theory fits our findings. Our results suggest that bacteria exposed to phage can either burst, or become lysogenic or recover from phage infection, according to the virulence of the phage particle and the resistance of the host it encounters.

MATERIALS USED

(1) Lemco broth. 1% Lemco in 1% peptone water with 0·5% sodium chloride at pH 7·6.

(2) Buffer. Phosphate buffer adjusted to pH 7·6.

(3) Lemco agar. Lemco broth solidified with 2% agar.

(4) CCY broth (Gladstone & Fildes, 1940).

(5) Indicator strain. *Sal. typhimurium* 1404.

(6) Phage A1 alpha lysate. The supernatant fluid of a centrifuged overnight broth culture of strain 1404/A1 which had been heated at 59° for 1 hr.

(7) Phage A1 beta lysate. Unless otherwise stated in the text, this was a culture of strain 1404 in Lemco broth which had been inoculated during the early logarithmic phase of growth, with a plaque from an alpha lysate spread lawn. After 18 hr. at 37° the culture was heated at 59° for 1 hr. and then centrifuged to clear.

RESULTS

Two kinds of nibbled colony

Indicator strain 1404, which had grown for 5 hr. in Lemco broth at 37° to a total count of about 5×10^8 organisms/ml., was diluted to about 2×10^8 organisms/ml. in broth; 2 ml. samples of this culture were added to 2 ml. volumes of phage suspension at concentrations varying from 2×10^8 to $1{\cdot}4 \times 10^9$ particles/ml. After 15 min. at 37° the mixtures were diluted to $1/10^5$ in buffer and 0·2 ml. amounts of the dilution sown on Lemco agar plates and allowed to soak into the agar. The plates were incubated at 37° for 18 hr. and the resulting colonies counted. Before the addition of phage a viable count was made of the culture used and the percentage survival rate, after treatment with phage, was calculated. The result of one such experiment is shown in Table 1. It is seen that as the phage : bacteria ratio increased so did the number of surviving organisms. At the same time there was an increase in the number of colonies showing central nibbling (Pl. 1, fig. 1). The number of colonies showing peripheral erosion rose to maximum at a ratio of phage to bacteria of 3 : 1 and fell off at ratios higher than this.

Table 1. *The results of exposing* Salmonella typhimurium 1404 *to varying amounts of phage A*1. *Mixture of phage and organisms made in broth and subsequently plated out for a viable count*

Ratio phage : bacteria	Bacterial survival rate (%). Mean of five plates	% of survivors showing central punctate nibbling	% of survivors showing peripheral erosion of colony
7	44·5	19	2·9
6	34·0	—	—
5	34·0	15	3·4
4	30·0	—	—
3	28·5	15	4·7
2	26·0	—	—
1	25·0	3·5	3·0

Single cell methods in tubes

A Lemco broth culture of *Sal. typhimurium* 1404 at about 5×10^8 organisms/ml. was diluted in broth to contain approximately 2×10^8 organisms/ml. The bacterial suspension was then added to an equal volume of phage beta lysate of known strength and the mixture left at 37° for 20 min. The mixture was then diluted in broth to give approximately 100 organisms/ml. A final dilution of 1/100 was made in ice cold broth to give approximately 1 organism/ml.; 0·5 ml. amounts of this dilution were tubed. After incubation overnight at 37° all the tubes which showed growth were heated for 60 min. in a water bath at 59°. With standard dropping pipettes delivering 36 drops/ml. drops were placed on lawns of the indicator strain and, after incubation overnight, a semi-quantitative titre of phage activity was read thus: 0 = no phage; + = about 100 or slightly more plaques/drop; + + = clear ring surrounding semi-confluent growth; + + + = clear ring surrounding confluent central growth.

Some of the tubes which showed no bacterial growth gave a positive phage titre. This was most probably due to the phage lysing the contained organism or organisms. Titration of such tubes yielded 1–50 plaques/drop. One could thus tell how many tubes contained phage particles.

Normally a lysogenic culture of strain 1404/A1, which has grown for 18 hr. at 37° in Lemco broth, gives a free phage titre of 10^4 particles/ml. On the other hand a phage-sensitive organism such as the indicator organism 1404 will give a titre of 10^8–10^{10} when grown with a free phage particle, or a lysogenic organism, in the tube. These figures correspond reasonably well to the + and + + + titres noted above.

Table 2. *Single cell study of* Salmonella typhimurium 1404 *exposed to phage A*1

Bacterial titre (viable)	$1{\cdot}8 \times 10^8$/ml.
Phage titre	$2{\cdot}9 \times 10^9$/ml.
Phage : bacteria ratio	16
Mean number of organisms/0·5 ml. dilution ...	0·45
Number of tubes inoculated with 0·5 ml. ...	427

Observed after 18 hr.	Expected	Found
No phage. No growth	273 (±33)	270
No phage. Growth	0	0
Growth. Phage +	.	113
Growth. Phage + +	.	1
Growth. Phage + + +	.	25
Phage increase. No growth	.	18

The result of an experiment of this kind is shown in Table 2. It will be seen that of those tubes which showed a phage increase together with growth of bacteria, 25 of 139 gave a + + + titre of phage. This could be due to one of several causes: (i) free unabsorbed phage in the presence of a non-lysogenic organism; (ii) a phage-infected and a sensitive organism being present in the same tube due to sampling chance; (iii) lysogenic and sensitive organisms growing in the same tube because the daughter cells of a phage-infected bacterium are not all lysogenic; (iv) a large number of cells having been induced to burst.

The third suggestion is probably correct for the following reasons:

(*a*) The use of a washed 18 hr. culture instead of a young broth culture of strain 1404 did not alter the ratio of + to + + + titres obtained, thus ruling out the factor of bacterial multiplication during exposure to phage.

(*b*) The presence of phage antiserum in buffer in the first dilution tube did not alter the proportion of + to + + + yields of phage in the experiment when bacteria are exposed to multiple phage infection.

(*c*) The distribution of number of organisms/tube seemed to follow fairly closely the expected Poisson distribution. This was detected from the expected and found values of tubes containing neither phage nor growth and from the distribution of the burst sizes for individual bacteria and multiple bacteria per tube.

(*d*) 'Induction' of lysis seemed unlikely to occur regularly.

Variation in phage virulence

Plaque size variation. Lawns made by spreading a culture of strain 1404 on agar plates and allowing it to soak into the agar, were spread with 0·1 ml. amounts of dilutions of alpha lysate, and similarly lawns were spread with 0·1 ml. of dilutions of lysates prepared by different methods described below. After overnight incubation at 37° the sizes of the plaques which had developed were measured on a plate microscope with an eyepiece micrometer. The experiments were arranged so that not more than 100 plaques developed on a plate. By this means overcrowding was prevented and measurements were more accurate. The mean plaque size varied from plate to plate even when the same lysate was used. The scatter in results was examined statistically. Table 3 shows that the scatter in plaque size of an alpha lysate was greater than that of a beta lysate, but that the plaque size for both lysates was scattered around a mean in the form of a fairly regular curve.

Table 3. *The plaque size scatter of phage A*1 *alpha lysate,* (*a*) *and* (*b*), *compared with phage A*1 *beta lysate,* (*c*) *and* (*d*)

	Eyepiece micrometer measurements (19=1 mm.)								
	Below 5	6–10	11–15	16–20	21–25	26–30	31–35	36–40	41–45
(*a*)	2	6	13	11	7	8	9	2	3
(*b*)	0	2	11	13	13	7	7	5	2
(*c*)	0	0	0	3	3	13	29	12	0
(*d*)	0	0	0	0	2	12	16	25	5

	(*a*)	(*b*)	(*c*)	(*d*)
Mean value	21·7	23·2	31·7	34·4
Variance	103	81	21	21

In Table 4 a comparison is given of the scatter in plaque size from alpha and beta lysates prepared in the CCY broth of Gladstone & Fildes and also from an ultraviolet induced lysate. The alpha lysate was a sintered-glass filtrate of a 3 hr. culture of strain 1404/A1; the beta lysate was from a culture of 1404 in CCY broth to which had been added one drop of alpha lysate, the mixture being incubated overnight and then sintered-glass filtered. The induced lysate was prepared as follows:

A 3 hr. culture of strain 1404/A1 at a concentration of about 10^7 organisms/ml. in CCY broth was pipetted into a Petri dish to a depth of 2 mm. This was exposed to ultraviolet light from a Hanovia lamp at a distance of 44 cm. for 2 min. After a further 90 min. incubation the lysate was filtered through a 5/3 sintered-glass filter. The phage titre was $6{\cdot}5 \times 10^5$ particles/ml. which is over 50 times higher than the titre one would normally find in an unirradiated culture. It will be seen from Table 4 that the plaques from an alpha lysate varied markedly in size and more so than those of the beta lysate. The plaque size scatter of an ultraviolet-induced lysate was nearer to that of an alpha lysate than it was to that of a beta lysate.

Table 4. *The plaque size scatter of phage A*1 *alpha lysate, beta lysate and ultraviolet-induced lysate*

		Eyepiece micrometer measurements (19 = 1 mm.)						
Lysate		11–15	16–20	21–25	26–30	21–35	36–40	41–45
Alpha	(*a*)	0	2	5	24	38	8	0
	(*b*)	0	4	0	5	21	29	25
Beta	(*c*)	0	0	5	18	58	59	3
	(*d*)	0	2	1	28	49	16	1
UVL	(*e*)	0	1	3	20	20	9	0
	(*f*)	0	1	2	4	15	18	6

	Mean	95% confidence limit plus or minus	Variance
(*a*)	30·66	1·04	20·34
(*b*)	36·23	1·38	41·73
(*c*)	34·25	0·66	16·48
(*d*)	31·98	0·79	15·84
(*e*)	30·70	1·20	19·80
(*f*)	34·60	1·60	30·81

Variation in lysate titres. The alpha and beta lysates used in the experiments detailed in Table 3 were diluted in broth so that, on the average, each 1 ml. of broth dilution contained 1 phage particle; 0·5 ml. amounts of these phage dilutions were tubed and to each tube was then added 2 ml. broth culture of indicator strain at a concentration of 10^6 organisms/ml. After overnight incubation all the tubes were heated at 59° to kill the bacteria and one drop from each tube was dropped on to lawns of indicator strain. After overnight incubation those tubes which were found to contain phage were titrated; the results are given in Table 5. The titres from those tubes which had contained a phage particle from an alpha lysate were lower than those from tubes which had contained a particle from a beta lysate; both sets of titrations showed a scatter.

Table 5. *A comparison of the titres obtained by isolation of single particles from alpha and beta lysates into cultures of indicator strain and incubating for* 18 *hr. Titres expressed as no. of phage particles/ml.*

Alpha lysate titres	Beta lysate titres
$1·66 \times 10^{10}$	$3·32 \times 10^{10}$
$1·18 \times 10^{10}$	$2·67 \times 10^{10}$
$8·4 \times 10^{9}$	$3·01 \times 10^{10}$
$2·3 \times 10^{10}$	$3·18 \times 10^{10}$
$2·25 \times 10^{10}$	$3·36 \times 10^{10}$
$1·52 \times 10^{10}$	$3·28 \times 10^{10}$
$1·32 \times 10^{6}$	$1·86 \times 10^{10}$

Variation in mean bacterial resistance due to change of medium

Plates were poured of Lemco agar and also of Lemco agar containing 10^8 phage particles/ml. agar mixture. After drying, four of each kind of plate were spread with dilutions in broth of cultures of strain 1404 grown overnight in: (*a*) 1 % peptone water, (*b*) Lemco broth, and (*c*) Koser's citrate medium. After overnight incubation at 37° colony counts were made and the percentage survival rates calculated (Table 6). It will be noted that organisms grown in Koser's citrate medium were more sensitive to phage than those grown in Lemco broth and these, in their turn, were more sensitive than those grown in peptone water. Colonies of strain 1404 picked from a Lemco agar plate and grown for 3 hr. in Lemco broth varied in sensitivity to phage but the degree of variation was irregular on sub-culture (Table 7).

Table 6. *Comparison of the percentage survival rates for* Salmonella typhimurium 1404, *grown on three different media, on exposure to phage A*1

A = colony counts on Lemco agar.
B = colony counts on agar containing 10^8 phage particles/ml.

Type of culture medium used					
Peptone water		Lemco broth		Koser's citrate	
A	B	A	B	A	B
Colony counts					
161	61	74	26	79	15
175	46	62	16	83	21
166	71	70	15	84	14
153	66	57	15	77	13
= 37%		= 27%		= 19%	

Table 7. *Comparison of the percentage survival rates of* Salmonella typhimurium 1404 *colonies on exposure to phage A*1 *in Lemco agar. Phage titre/ml. agar mixture* = 10^8

Culture no. ...	1	2	3	4	5	6	7	8	9
Colony counts on five agar plates	1082	804	944	807	692	809	737	972	680
Colony counts on five phage agar plates	226	139	177	152	150	235	174	276	184
% survivors	20·9	17·3	18·7	18·9	21·7	29·0	23·6	28·4	27·0
95% confidence limits plus or minus	1·0	2·8	2·0	1·8	3·1	3·2	4·0	5·1	1·2

DISCUSSION

Two distinct types of colonial abnormality due to phage action were observed. First, a type with a nibbled periphery; this probably arises through the action of free phage coming into contact with the edge of a growing non-lysogenic colony, thereby producing erosion. Second, a colony with a central punctate type of nibbling; the number of these increases as the number of lysogenic colonies increases. When most of the colonies on the plate are lysogenic, the proportion of centrally nibbled colonies is about 20 %. This proportion remains fairly constant even with a high infecting dose of phage. Phage antiserum used to neutralize excess phage after exposure of the bacteria does not alter the percentage of centrally nibbled colonies, neither does the age of the bacterial culture used. The central nibbling is not due to the production of a phage variant arising in a lysogenic organism. Centrally nibbled colonies picked from a plate to broth and incubated overnight give a free phage titre of 10^5–10^6 phage particles/ml. On the other hand peripherally nibbled colonies similarly treated yield 10^7–10^8 particles/ml. Lysogenic and non-lysogenic cultures can be recovered from both types of colony. From the results given in Table 2 it will be seen that at a high multiplicity of phage infection, a proportion of the tubes which grow shows a higher free phage titre than the rest. This proportion is about 20 %.

On the basis of these facts it seems that a single organism exposed to phage can produce a mixed progeny of organisms, some of which are lysogenic and some of which are non-lysogenic and phage-sensitive. This seems to occur at the inception of the symbiotic state and in some 20 % of cases. The established lysogenic culture does not behave in this way.

It is known that bacteria exposed to multiple infection by phage A1 are more likely to survive as lysogenic organisms than if they are exposed to about 1·5 phage particles/organism. Boyd accounts for this (1951, *b*) by suggesting that a phage beta lysate is composed of two types of particle, the alpha particle being able to produce the symbiotic state even if the organism is infected by one or more beta particles at the same time.

The results of our experiments also suggest that symbiosis is the dominant state; if an organism contacts a phage of weak lytic ability it is protected from highly active phage particles. The production of symbiosis is not due to the action of alpha particles but due to particles in the lysate with little ability to lyse the organism they invade. If the bacteria were all of the same sensitivity this would be tantamount to saying that all phage particles having less than a given lytic ability could be regarded as alpha particles and all those with greater lytic ability as beta particles. However, we have shown also that bacteria vary in their susceptibility to lysis depending on previous conditions of growth. One can therefore postulate that the establishment of symbiosis is a host-parasite relationship for each phage particle and for each bacterium the phage invades; in other words a phenomenon similar to that occurring in other forms of virus disease. It was D'Herelle (1926) who first suggested that the establishment of a symbiotic state was due to a balance of individual phage particle virulence with individual bacterial resistance and the present experiments help to substantiate this view.

We should like to express our thanks to Dr D. W. Henderson for his help in preparing this paper and during the experimental work. Our thanks are also due to Professor E. T. C. Spooner and Brigadier J. S. K. Boyd for discussing the work with us. Acknowledgment is made to the Chief Scientist, Ministry of Supply for permission to publish this paper.

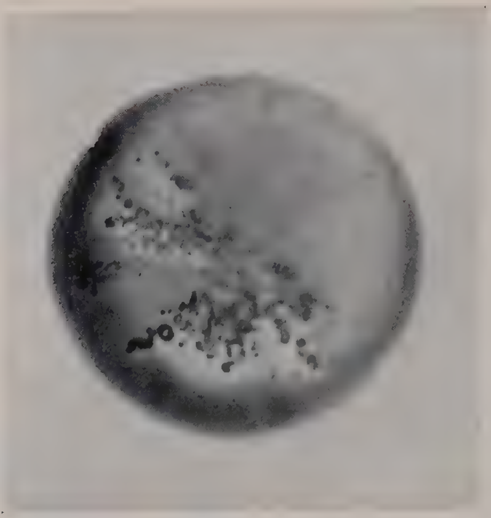


Fig. 1

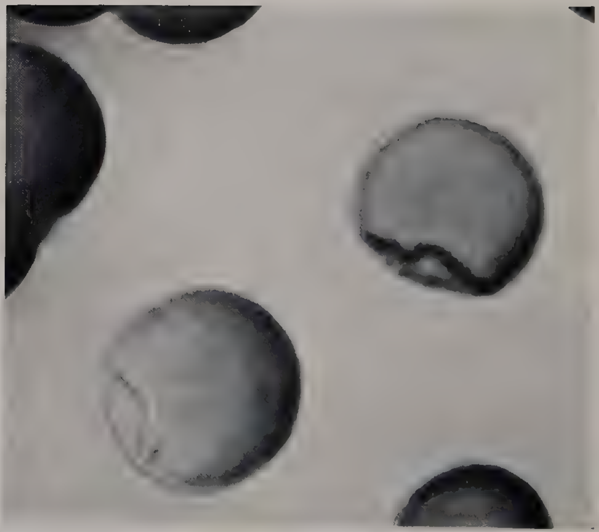


Fig. 2

W. R. PARRY & J. EDWARDS—INDUCTION OF LYSOGENESIS IN *SAL. TYPHIMURIUM*. PLATE 1

REFERENCES

Boyd, J. S. K. (1950). The symbiotic bacteriophage of *Salmonella typhimurium. J. Path. Bact.* **62**, 501.

Boyd, J. S. K. (1951*a*). Observations on the relationship of symbiotic and lytic bacteriophage. *J. Path. Bact.* **63**, 445.

Boyd, J. S. K. (1951*b*). 'Excessive dose' phenomenon in virus infections. *Nature, Lond.* **167**, 1061.

D'Herelle, F. (1926). *Le Bactériophage et son comportement.* Paris: Masson et Cie.

Gladstone, G. P. & Fildes, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.

Lwoff, A. & Gutmann, A. (1950). Recherches sur un *Bacillus megatherium* lysogène. *Ann. Inst. Pasteur*, **78**, 711.

EXPLANATION OF PLATE

Fig. 1. The central punctate type of colonial nibbling. The colony consists of a mixture of lysogenic and non-lysogenic phage sensitive organisms.

Fig. 2. Late colonial nibbling due to the action of phage A1.

(*Received* 30 *April* 1953)

Clarke, P. H. (1953). *J. gen. Microbiol.* 9, 350–352.

Growth of Streptococci in a Glucose Phenolphthalein Broth

By PATRICIA H. CLARKE

National Collection of Type Cultures, Colindale Avenue, London, N.W. 9

SUMMARY: A liquid medium buffered at pH 9·6 is described which is modified from that of Shattock & Hirsch (1947) by the addition of phenolphthalein and in the method of preparation. Its use in distinguishing streptococcal species was investigated.

Shattock & Hirsch (1947) described a liquid glycine-buffered medium for testing the ability of streptococci to grow at pH 9·6, one of the criteria found useful by Sherman & Stark (1934) and Shattock & Mattick (1943–44) for distinguishing *Streptococcus faecalis* from *Strep. lactis*. This medium was used by Shattock (1949) and by Sharpe & Shattock (1952) in studies on group D streptococci and *Strep. lactis*. It was thought that if a suitable indicator could be added to the medium it might be possible to dispense with the electrometric measurement of pH value, and as streptococci readily produce acid from glucose, growth would be accompanied by a change in colour of the indicator. Phenolphthalein with a pK of 9·7 was selected.

PREPARATION OF MEDIUM

The medium was prepared as described by Shattock & Hirsch (1947) with the modifications discussed above. Only 100 ml. were prepared at a time, sufficient for testing about sixteen strains.

(1) Stock Lemco broth pH 7·0 + 1 % w/v glucose.

(2) Glycine buffer: glycine, 0·75 g.; NaCl, 0·585 g., were dissolved in 100 ml. freshly boiled distilled water. To 60 ml. glycine + NaCl were added 40 ml. 0·1 N-NaOH to make solution (2).

(3) Set of buffer solutions pH 9·5, 9·6, 9·7, 9·8, 9·9. Clark & Lubs borate buffers were used as they were less likely to become contaminated. 20 ml. were prepared at each pH value, to which 0·1 ml. 0·2 % phenolphthalein was added. After mixing, the solutions were poured into bijou bottles (*c.* 5 ml.) and the caps screwed tightly to exclude air. They remained unchanged for at least 2 weeks.

To 90 ml. solution (1) were added 10 ml. solution (2) and 0·5 ml. phenolphthalein, and the reaction adjusted to pH 9·9 with N-NaOH by matching with the buffers in the bijou bottles, but any standard method for matching pH indicators may be used. No difficulty was experienced in matching the colours but the accuracy obtained was not greater than ± 0·25 pH unit. The medium was kept overnight in a refrigerator and then passed through a Seitz filter. Occasionally precipitation continued after filtration, but this was ignored as it did not affect the results. The sterile medium was distributed into bijoux bottles leaving as little air space as possible. The bottles were incubated overnight to check sterility and on the next day inoculated from 24 hr. agar or serum agar slope cultures. The colour of the medium at this stage is a definite clear pink. Any bottles in which the pH value has fallen to pH 9·2 or less will appear a pale orange pink colour and can be discarded. This makes possible a check on each individual bottle before inoculation. Uninoculated bottles and known positive and negative organisms were included in each set of tests. Tests were carried out at 37° and 30°. Controls were prepared of the buffered medium adjusted to pH 7·0 with bromthymol blue as indicator, and without indicator. Tubes of glucose peptone water as used for fermentation tests were incubated concurrently in a number of the tests.

Only a small number of strains has been tested; the group D and group N strains maintained in the N.C.T.C., strains of *Strep. lactis* kindly provided by Dr Shattock from her collection, and representative N.C.T.C. strains of the other serological groups of streptococci, and strains of *Aerococcus viridans* (Williams, Hirch & Cowan, 1953).

RESULTS

The medium was simple to prepare and proved quite satisfactory in use. The reaction after filtration varied between pH 9·5–9·6. Out of twelve batches of medium only an occasional bottle gave a pH value of 9·2 or less, appearing orange-pink instead of a clear definite pink colour. Two *Strep. lactis* strains tested in media at about pH 9·2 were able to grow whereas no growth occurred in the medium at pH 9·5–9·6.

The results were clear-cut with the *Strep. lactis* and *Strep. faecalis* strains tested at 30° and 37°. All the *Strep. faecalis* strains grew with acid production in 24 hr. and none of the *Strep. lactis* in 24 or 48 hr. in repeated tests (Table 1). In the medium at pH 7·0, with or without bromthymol blue and in glucose peptone water, both species grew and produced acid in 24 hr. None of the strains of *Strep. bovis* grew at pH 9·6. For *Strep. durans* this pH is obviously near the critical limits for growth. Shattock & Hirsch (1947) found that nine out of eleven strains grew at pH 9·6. In repeated tests two of our six strains grew

Table 1. *Ability of* Streptococcus *species to grow in a medium at* pH 9·6

Results after incubation for 24 hr.

		No. of strains	
Serological group	Species	Growing at pH 9·6	Not growing at pH 9·6
Group D	*Strep. faecalis*	14	0
	Strep. liquefaciens	7	0
	Strep. zymogenes	3	0
	Strep. durans	2	4
	Strep. bovis	0	6
	Unclassified	2	2
Group N	*Strep. lactis*	0	14
Groups A, B, C, E, G, M	—	0	28
—	*Aerococcus viridans*	10	0

and produced acid in 24 hr. at pH 9·6, three grew and produced acid in 48 hr. but not in 24 hr., and one strain failed to grow in 3 days. All ten *Aerococcus viridans* strains grew at pH 9·6.

These results agree with those obtained by Shattock & Hirsch. The simplified method of preparation of this medium may contribute to the more general use of this test for growth at pH 9·6.

REFERENCES

SHARPE, M. E. & SHATTOCK, P. M. F. (1952). The serological typing of group D streptococci associated with outbreaks of neonatal diarrhoea. *J. gen. Microbiol.* **6**, 150.

SHATTOCK, P. M. F. (1949). The streptococci of Group D; the serological grouping of *Streptococcus bovis* and observations on serologically refractory group D strains. *J. gen. Microbiol.* **3**, 80.

SHATTOCK, P. M. F. & HIRSCH, A. (1947). A liquid medium buffered at pH 9·6 for the differentiation of *Streptococcus faecalis* from *Streptococcus lactis*. *J. Path. Bact.* **59**, 495.

SHATTOCK, P. M. F. & MATTICK, A. T. R. (1943–44). The serological grouping of *Streptococcus lactis* (Group N) and its relationship to *Streptococcus faecalis*. *J. Hyg., Camb.* **43**, 173.

SHERMAN, J. M. & STARK, P. (1934). The differentiation of *Streptococcus lactis* from *Streptococcus faecalis*. *J. dairy Sci.* **17**, 325.

WILLIAMS, R. E. O., HIRCH, A. & COWAN, S. T. (1953). *Aerococcus*, a new bacterial genus. *J. gen. Microbiol.* **8**, 475.

(*Received* 18 *May* 1953)

No. 2, *Volume* 9 *of the Journal of General Microbiology was issued on* 8 *October* 1953.

BERGERSEN, F. J. (1953). *J. gen. Microbiol.* 9, 353–356.

Cytological Changes induced in *Bacterium coli* by Chloramphenicol

BY F. J. BERGERSEN

Department of Bacteriology, University of Otago Medical School, Dunedin, New Zealand

SUMMARY: Under certain conditions chloramphenicol influences the growth and division of *Bacterium coli* so that abnormal forms are produced. These include long and sometimes pointed filaments and branched forms which seem to arise from organisms with abnormal growing points. Changes in morphology are preceded by changes in nuclear structure. The processes described occur only within critical limits of concentration of the antibiotic, and these limits vary when different strains of *Bacterium coli* are used.

It was found by Knaysi (1930) that basophilic granules occurred in the cell periphery of *Bacillus subtilis* at the site of development of transverse membranes. Similar granules have also been shown by the author in *B. megaterium* (Bergersen, 1953). Bisset (1951) described regions of active growth in Gram-negative bacilli, in which the structures of the cell envelope are laid down. These show as deeply stained areas with basic dyes and have been termed 'growing points'. Fleming, Voureka, Kramer & Hughes (1950) demonstrated cytological changes induced in *Proteus vulgaris* by penicillin. More recently, Pulvertaft (1952) described characteristic changes in *Bacterium coli* grown on media containing chloramphenicol. Evidence that chloramphenicol affects the nuclear material was presented by Voureka (1952), who showed heritable changes produced in a staphylococcus after treatment with the antibiotic. The present work contains additional evidence that chloramphenicol acts on nuclear processes, and demonstrates the possibility that abnormal growing points have a role in the production of aberrant forms.

MATERIALS AND METHODS

The effects of chloramphenicol were tested against eight freshly isolated faecal strains of *Bact. coli*. These were grown in 5 ml. amounts of nutrient broth in 20 ml. screw-cap bottles. Centrifuged deposits from cultures of various ages were smeared on coverslips, fixed in osmic acid vapour and dried in air at room temperature. Cytoplasmic structures were examined, using the HCl+Giemsa method of Robinow (1942). Cell walls were stained by his tannic acid-crystal violet method and by 0·5 % Victoria blue 4R (Gurr). All preparations were examined in water mounts.

Chloramphenicol was added to cultures in concentrations which slightly inhibited growth after 12 hr. incubation at 37°; for most of the strains used this concentration was 2·5 μg./ml. Two strains were completely inhibited by this concentration, and 1·0 μg./ml. gave the characteristic morphological changes. In a particularly resistant strain these changes were produced with 25 μg./ml.

OBSERVATIONS

Untreated cultures contained typical organisms 2–4μ. long and 0·5μ. in diameter. Cell membranes were stained a darker pink than the cytoplasm by the HCl-Giemsa method, and growing points appeared as granules at the poles and at the transverse septa of dividing cells. The nuclear bodies stained a deep blue (Pl. 1, fig. 1). Seven minutes hydrolysis in N-HCl was sufficient to produce satisfactorily stained preparations from untreated cultures, but longer periods, in proportion to the time in contact with the antibiotic, were required for those treated with chloramphenicol.

After treatment for 8 hr. the organisms were slightly larger than those of the control cultures, and their nuclear material was arranged in irregular bars which were often asymmetrically distributed in the cells. There appeared to be little or no cell division in these cultures and only polar growing points were seen (Pl. 1, fig. 2).

Organisms treated for 16 hr. were further enlarged and the polar growing points were prominent and well stained. Approximately one organism in twenty had additional granules which often protruded from the cell outlines and which were apparently identical to the granules of the polar growing points. These granules were usually in the lateral part of the cell membrane towards one pole of the cell. Nuclear bodies were enlarged and in pairs (Pl. 1, figs. 3–6).

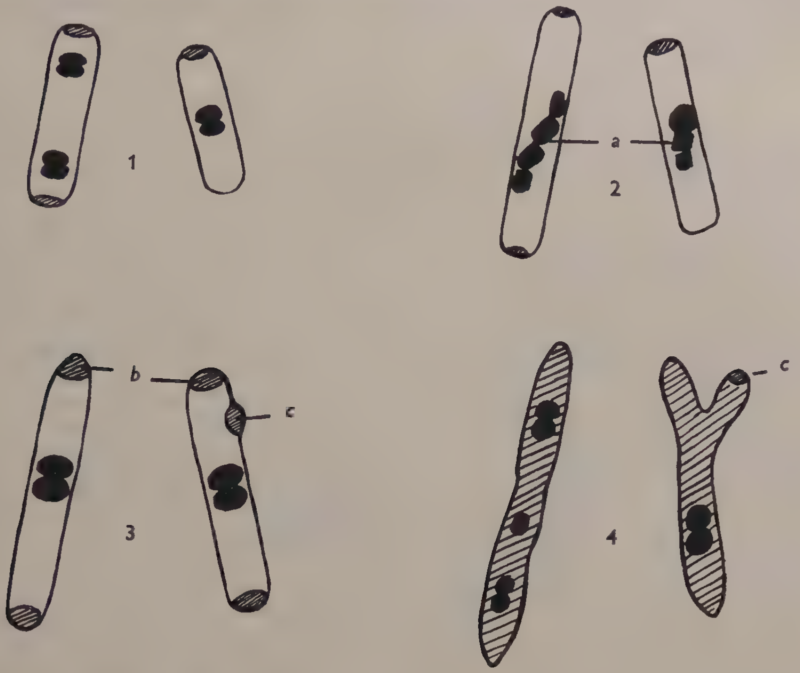
After 22–28 hr. incubation the organisms were enormously enlarged, difficult to hydrolyse, and, in many cases, sharply pointed. In these preparations approximately one organism in twenty appeared to be branched. When cell-wall stains were used these giant forms were seen to have few transverse septa and the branched forms possessed continuous cell walls (Pl. 1, figs. 7–10). After further incubation the branched forms were larger and stained poorly, having the appearance of dead cells (Pl. 1, fig. 11).

A 16 hr. culture containing cells with abnormal growing points was centrifuged and resuspended in several changes of broth to remove the antibiotic. After 12 hr. incubation, HCl+Giemsa preparations showed that the resulting culture was returning to normal (Pl. 1, fig. 12).

DISCUSSION

From this work it seems that chloramphenicol exerts an effect on nuclear division in *Bact. coli.* Abnormal appearances of the nuclear material are among the first cytological changes observed. Extra granules which resemble growing points in their staining reactions then make their appearance in some cells and polar growing points become prominent in all of them. Cell proliferation in these regions may result in the branched forms and in the long, pointed organisms, as illustrated in the diagrams, Figs. 1–4. Up to a certain stage these changes seem to be reversible, as removal of the antibiotic results in normal cultures, while further incubation with the antibiotic results in loss of viability.

Pulvertaft's description of the giant cells obtained from *Bact. coli* treated with chloramphenicol corresponds fairly closely with this work. His failure to observe branching may have been due to the use of different antibiotic concentrations, to the use of solid media, to the different duration of the experiments, or to combinations of these factors.



Figs. 1–4. Diagrams of the changes in a strain of *Bact. coli* grown in the presence of 2·5 μg. chloramphenicol/ml. Fig. 1. Typical untreated cells. Fig. 2. After 8 hr. treatment showing enlargement and nuclear changes (*a*). Fig. 3. After 16 hr. treatment showing prominent polar growing points (*b*) and an extra growing point (*c*). Fig. 4. Giant forms after 22 hr. treatment—the cell envelopes are dense and the cytoplasm basophilic. Some show branching as at (*c*).

Branching was only seen in some of the treated organisms and this fraction seemed to correspond to the proportion of cells showing extra growing points at an earlier stage. These extra growing points may be part of the normal process of cell division, but this cannot proceed in the normal way because of the affected nucleus. Asymmetrical growth may result, and in some cases this may lead to outgrowths of the cell to give a branched form.

REFERENCES

Bergersen, F. J. (1953). A probable growth cycle in *Bacillus megaterium*. *J. gen. Microbiol.* 9, 26.

Bisset, K. A. (1951). The development of the surface structures in dividing bacteria. *J. gen. Microbiol.* 5, 155.

Fleming, A., Voureka, A., Kramer, I. R. H. & Hughes, W. H. (1950). The morphology and motility of *Proteus vulgaris* and other organisms cultured in the presence of penicillin. *J. gen. Microbiol.* 4, 257.

Knaysi, G. (1930). Cell structure and cell division of *B. subtilis*. *J. Bact.* 19, 113.

Pulvertaft, R. J. V. (1952). The effect of antibiotics on growing cultures of *Bacterium coli*. *J. Path. Bact.* **64**, 75.

Robinow, C. F. (1942). A study of the nuclear apparatus of bacteria. *Proc. Roy. Soc.* B, **130**, 299.

Voureka, A. (1952). Induced variations in a penicillin-resistant staphylococcus. *J. gen. Microbiol.* **6**, 352.

EXPLANATION OF PLATE

Fig. 1. Cells from an untreated 8 hr. broth culture of *Bact. coli* showing paired nuclear bodies and polar growing points. HCl + Giemsa, × 2000.

Fig. 2. Cells from an 8 hr. culture treated with 2·5 μg. chloramphenicol/ml. HCl + Giemsa, × 2000.

Figs. 3, 4. Cells from a 16 hr. treated culture showing prominent polar growing points. HCl + Giemsa, × 3000.

Figs. 5, 6. Cells from a 16 hr. treated culture showing polar and sub-polar extra growing points. HCl + Giemsa, × 3000.

Fig. 7. A branched organism from a 22 hr. treated culture. Giemsa, × 3000.

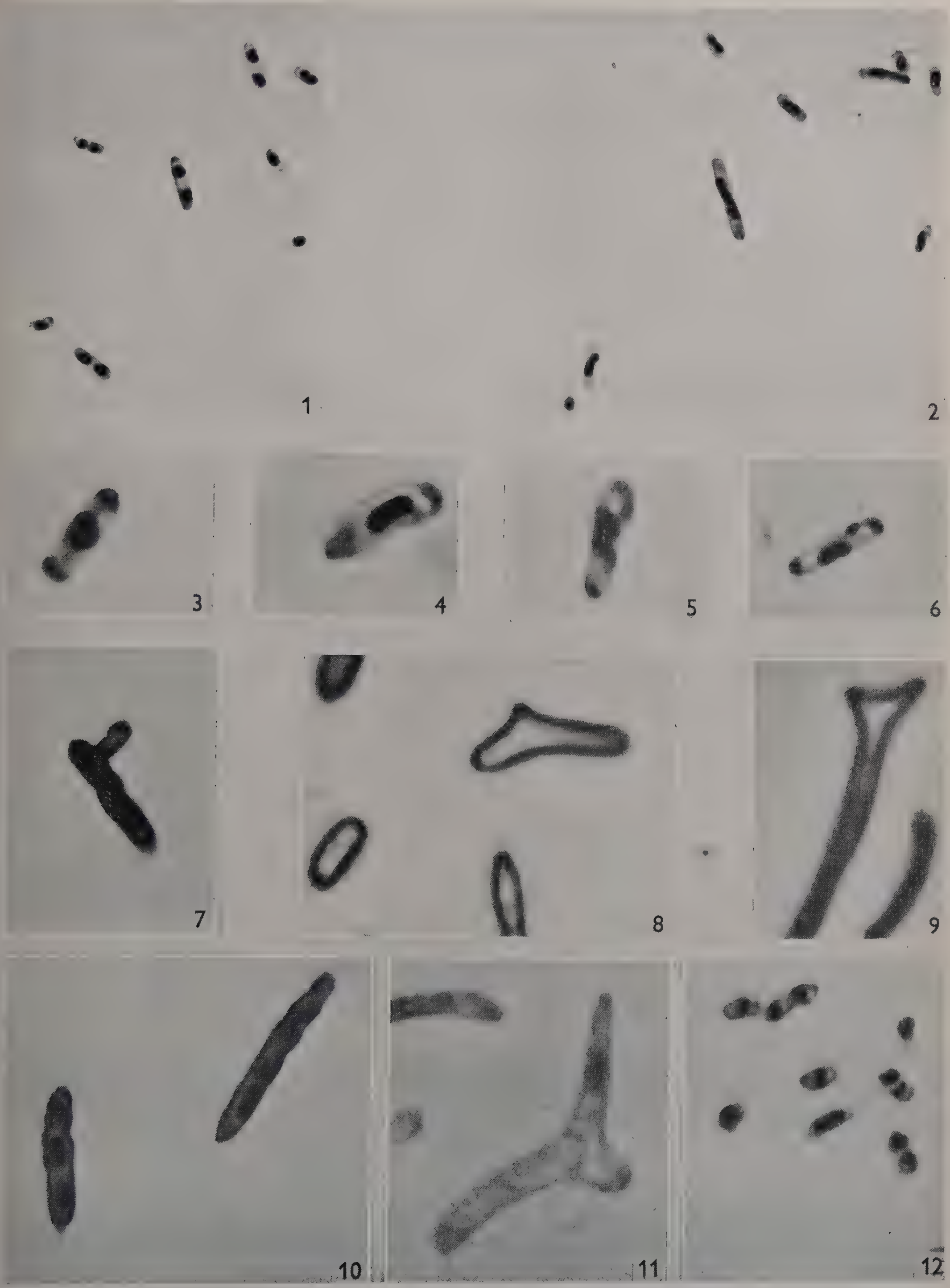
Figs. 8, 9. Branching organisms stained to show the cell wall. Tannic acid-crystal violet, × 3000.

Fig. 10. Long pointed forms from a 22 hr. treated culture. HCl + Giemsa, × 3000.

Fig. 11. A branched form from a 36 hr. treated culture. HCl + Giemsa, × 3000.

Fig. 12. Cells from a 16 hr. treated culture washed free of antibiotic. Normal morphology. HCl + Giemsa, × 3000.

(*Received 2 February* 1953)


1
2
3
4
5
6
7
8
9
10
11
12

F. J. Bergersen—Cytological changes in *Bacterium coli*. Plate 1

VINCENT, J. M. & WATERS, L. M. (1953). *J. gen. Microbiol.* 9, 357–370.

The Influence of the Host on Competition amongst Clover Root-nodule Bacteria

BY J. M. VINCENT AND L. M. WATERS

Agricultural Microbiology Laboratory, Faculty of Agriculture,
University of Sydney, N.S.W., Australia

SUMMARY: Serological typing has been used to study competition between five strains of *Rhizobium trifolii* in growth and nodulation with four species of host. Although a proportion of non-reacting antigenic variants appears to have been encountered, the serological method remains uniquely suitable for studies of this kind.

Irrespective of host species or level of inoculum the population of bacteria in the tube of seedling agar approached a maximum of approximately 4×10^6 viable cells per ml. No consistent relationship was found between host species and the proportion of the several strains of rhizobia in the population outside the plant. The relative frequency of strains was, however, markedly affected by level of total inoculum and time of growth after inoculation. Some strains grew better at first but reached a lower maximum than others which fared better at higher population levels, whether from a heavier inoculum or prolonged growth. The population on the root surface itself failed to reflect any marked host influence in the balance between strains.

The proportions of strains found in the nodules were unrelated to their representation in the root's external environment. Each host exercised a specific selective effect in this regard.

Factors which determine the balance between strains of root-nodule bacteria (*Rhizobium* species) that are in competition with each other for growth and nodulation are likely to be of considerable practical and theoretical significance. Nitrogen fixation by a legume growing in a soil containing parasitic rhizobia will depend on the ability of an effective strain to compete with the ineffective bacteria. This will be true whether the effective strain is introduced as inoculum or is already part of the local bacterial flora. This aspect of the legume symbiosis is also interesting from an ecological point of view. The technique of serological typing opens up possibilities for investigating the effect of host species on the competitive balance between rhizobial strains. This account deals with our experience with four species of clover, each associated with five strains of clover nodule bacteria (*Rh. trifolii*).

METHODS

Hosts. Trifolium pratense L. (red clover), *T. incarnatum* L. (crimson clover), *T. repens* L. (white clover) and *T. subterraneum* L. (subterranean clover). All seed has been surface sterilized with ethanol and 0·2% (w/v) mercuric chloride and washed with several changes of sterile water. All uninoculated controls remained free of nodules.

Growth medium. Nitrogen-free seedling agar of the following composition (%, w/v): $CaHPO_4$, 0·1; K_2HPO_4, 0·02; $MgSO_4.7H_2O$, 0·02; NaCl, 0·02; $FeCl_3$, 0·01; agar 0·8; pH adjusted to pH 6·5 with NaOH; 10 ml. agar deeps in 6 × ⅝ in. test tubes.

Organisms. The source and nitrogen-fixing ability of each strain is given in Table 1. They were all serologically distinguishable.

Table 1. *Details of bacterial strains*

Collection number	Source	Effectiveness* with *T. pratense*	*T. repens*	*T. incarnatum*	*T. subterraneum*
36	*T. repens*	*e*	E	E	E
157	*T. repens*	E	E	I	I
204	*T. repens* (culture collection)†	E	E	I	I
284	*T. glomeratum*	*i*	E	E	E
297	*T. incarnatum*	*i*	E	E	E

* Grading based on several tests: E, highly effective; I, markedly ineffective; *e* and *i* are corresponding intermediate states.

† 'Clover F' clone from Dr H. G. Thornton, F.R.S., Rothamsted.

Setting up the test. Surface-sterilized seeds were allowed to germinate for 2 days in a sterile Petri dish, and well-developed seedlings planted singly in each tube of agar medium; 0·25 ml. mixed and diluted suspension of the five bacterial strains was added immediately to each tube.

Four levels of inocula were used: M_1, M_2, M_3 and M_4 of increasing tenfold concentration. Viable counts of the suspensions used in the preparation of inocula permitted the number of each strain to be determined. The numbers for M_1 (per tube of 10 ml. agar) are given below:

Strain	No. of viable cells
36	20
157	30
204	70
284	20
297	20
Total	160

Number of rhizobia in the root's external environment. The whole contents of the tube was shaken vigorously 25 times with 100 ml. sterile water to give a first dilution, *c.* 1/10. Subsequent dilutions and plate counts were set up in the usual way.

Estimation of proportion of each strain in the external environment. Twenty separated colonies conforming to the characteristics of rhizobium, but otherwise taken randomly, were picked separately to yeast mannitol agar slopes and the growth typed by testing a suspension (made up in 0·9% saline and heated at 100° for 30 min. to destroy the less specific flagellar antigens) against the range of sera at dilution 1/100. Control suspensions without serum were set up at the same time to detect unstable antigen.

Typing-nodule isolates. Six-week-old plants were removed aseptically from the agar and rinsed in sterile water, so that the suspensions could be used to estimate the proportions of the various strains in the root environment at that stage. The whole root surface was then sterilized with ethanol and 0·1 % (w/v) $HgCl_2$ for 2 min., washed in six changes of sterile water, and the contents of separate nodules streaked on plates of yeast mannitol agar. As far as possible, ten nodules were taken from each plant, the position of each relative to the crown of the plant having been recorded. The culture for serological typing was then developed from a characteristic and well-separated colony.

Presentation of results. Original data are given in full. In considering the results it is more convenient to express the proportions of each strain as a percentage of the total, but χ^2 tests, on which conclusions as to significance have been based, have all been calculated on actual numbers.

RESULTS

Total viable population at 3 and 10 days

Table 2 gives the logarithms of viable counts for duplicate tubes of each treatment. Generation time and total growth at 10 days are little, if at all, affected by the species of host. The former is consistently shorter for the earlier time interval and at the lower inoculum levels. By 10 days all treatments have reached approximately the same level: 40×10^6 per tube. The lowest inoculum (M_1) reached this level by 12 days, the only occasion of testing.

Ratio of strains in the tube population

The full data are set out in Tables 3 and 4.

The large proportion of isolates that fail to react with any of the testing sera calls for some comment. A plant test gave nodulation with all but one out of twenty-two such cultures from 3-day material. They cannot, therefore, be dismissed as non-rhizobial contaminants. Moreover, re-isolates from these nodules were all identified as one or other of several of the strains in these proportions: strain 36, 1; strain 157, 2; strain 204, 5; strain 284, 13. The application of this ratio to the total 3-day negative reactors appears reasonable. Whether it can be applied to the more numerous 10-day negative reactors is more doubtful, and data will be considered with and without this adjustment.

Agreement between duplicate tubes. It will be seen from Table 3 that there is fair to good agreement between the contents of duplicate tubes. The distribution of culture types, including the unstable and negative cultures, was tested by χ^2 for each pair of duplicate tubes. This gave twenty-four series of χ^2 each with 6 D.F. Only one of the series (3 days, M_3, white clover) gave a significant difference between duplicate tubes ($\chi^2 = 16{\cdot}1$; $P = 0{\cdot}02 - 0{\cdot}01$). The remaining χ^2 ranged from 2·2 to 12·2 (P from 0·9 to 0·10 – 0·05). Total χ^2 (144 D.F.) was 166·8 ($P = 0{\cdot}2 - 0{\cdot}1$).

The variation between duplicate tubes for strain 157, using all comparisons available in Table 3, with minor adjustments to bring all cases to the basis of a total of 20 per tube, gave a standard deviation for each tube of ± 2·0.

Remaining calculations have been based on the totals of duplicate tubes except in the subsidiary test with inoculum M_1 (Table 4) where duplicates were not available.

Table 2. *Growth data on seedling agar*

		Log numbers/tube (10 ml. medium)					Mean generation time (hr.)				
Inoculum level*	Time (days)	Red clover	White clover	Crimson clover	Subterranean clover	Mean	Red clover	White clover	Crimson clover	Subterranean clover	Mean
M_2 (3·20)	3	6·18	6·04	5·46	5·22						
		5·68	6·30	5·64	5·75						
		5·93	6·17	5·55	5·48	5·78	7·9	7·3	9·2	9·5	8·4
	10	7·32	7·29	7·31	7·47						
		7·49	7·83	7·76	7·75						
		7·40	7·56	7·54	7·61	7·53	17·2	16·5	16·6	16·3	16·6
M_3 (4·20)	3	6·64	6·94	7·15	6·35						
		6·46	6·15	6·72	6·14						
		6·55	6·55	6·94	6·25	6·57	9·2	9·2	7·9	10·5	9·1
	10	8·05	7·41	7·54	7·86						
		8·06	7·79	8·06	8·25						
		8·06	7·60	7·80	8·06	7·88	18·7	21·2	20·0	18·7	19·6
M_4 (5·20)	3	7·10	7·22	7·27	7·12						
		7·16	7·19	7·25	6·95						
		7·13	7·20	7·26	7·03	7·16	11·2	10·8	10·5	11·8	11·0
	10	7·46	7·24	7·18	7·43						
		7·57	7·41	7·41	7·60						
		7·51	7·33	7·30	7·52	7·41	31·2	33·8	34·2	31·0	32·5
Average	3	6·54	6·66	6·58	6·25	6·51	9·2	8·8	9·1	10·5	9·3
	10	7·66	7·49	7·54	7·73	7·54	20·8	21·8	21·5	21·0	21·5

* Log no. of organisms/tube.

Table 3. *Proportion of strains in growth outside the host plant (three inoculum levels, two occasions)*

		Number reacting															
		At 3 days as								At 10 days as							
Inoculum level	Clover host	36	157	204	284	297	Un-stable	Neg.	Total	36	157	204	284	297	Un-stable	Neg.	Total
M_2	Red	0	9	7	2	1	0	0	19	0	1	6	2	6	0	5	20
		1	4	4	1	1	0	4	15	0	1	2	2	9	0	6	20
	Total	1	13	11	3	2	0	4	34	0	2	8	4	15	0	11	40
	White	4	5	8	2	0	0	0	19	1	2	7	1	2	0	7	20
		0	10	4	3	0	0	3	20	0	2	1	3	4	0	9	19
	Total	4	15	12	5	0	0	3	39	1	4	8	4	6	0	16	39
	Crimson	2	11	1	2	0	0	3	19	0	4	1	4	4	0	7	20
		1	10	2	5	2	0	0	20	3	2	1	3	7	0	3	19
	Total	3	21	3	7	2	0	3	39	3	6	2	7	11	0	10	39
	Subterranean	1	3	9	6	0	0	0	19	0	4	0	0	9	2	5	20
		1	3	9	1	2	0	3	19	0	5	1	0	6	0	6	18
	Total	2	6	18	7	2	0	3	38	0	9	1	0	15	2	11	38
Total, M_2		10	55	44	22	6	0	13	150	4	21	19	15	47	2	48	156
M_3	Red	0	3	3	2	3	0	4	15	0	0	5	0	8	1	5	19
		0	6	6	2	3	0	3	20	0	1	1	0	7	0	11	20
	Total	0	9	9	4	6	0	7	35	0	1	6	0	15	1	16	39
	White	1	9	2	3	2	0	2	19	0	2	1	0	7	0	10	20
		0	2	7	2	1	0	8	20	0	1	0	5	4	1	9	20
	Total	1	11	9	5	3	0	10	39	0	3	1	5	11	1	19	40
	Crimson	0	5	7	1	0	1	1	15	0	4	0	3	10	0	3	20
		0	4	6	4	2	0	4	20	1	4	4	4	2	1	4	20
	Total	0	9	13	5	2	1	5	35	1	8	4	7	12	1	7	40
	Subterranean	1	3	3	5	4	0	0	16	0	2	2	4	5	0	6	19
		0	8	3	1	2	0	5	19	1	2	7	4	1	1	4	20
	Total	1	11	6	6	6	0	5	35	1	4	9	8	6	1	10	39
Total, M_3		2	40	37	20	17	1	27	144	2	16	20	20	44	4	52	158

Table 3 (*continued*)

		Number reacting															
		At 3 days as								At 10 days as							
Inoculum level	Clover host	36	157	204	284	297	Un-stable	Neg.	Total	36	157	204	284	297	Un-stable	Neg.	Total
M_4	Red	0	6	3	5	3	0	2	19	1	3	0	1	10	0	5	20
		0	1	2	4	4	1	4	16	0	0	0	0	9	0	10	19
	Total	0	7	5	9	7	1	6	35	1	3	0	1	19	0	15	39
	White	1	4	4	3	4	0	3	19	0	3	1	2	4	0	9	19
		1	3	3	10	0	0	2	19	0	2	0	0	4	0	14	20
	Total	2	7	7	13	4	0	5	38	0	5	1	2	8	0	23	39
	Crimson	1	2	6	1	4	1	5	20	0	1	0	0	7	1	9	18
		0	4	1	3	4	0	7	19	1	2	1	0	4	0	12	20
	Total	1	6	7	4	8	1	12	39	1	3	1	0	11	1	21	38
	Subterranean	0	8	5	1	2	0	3	19	1	0	1	0	7	0	10	19
		0	3	2	6	3	1	5	20	0	0	3	0	9	0	8	20
	Total	0	11	7	7	5	1	8	39	1	0	4	0	16	0	18	39
Total, M_4		3	31	26	33	24	3	31	151	3	11	6	3	54	1	77	155
Grand totals		15	126	107	75	47	4	71	445	9	48	45	38	145	7	177	469

Table 4. *Incidence of strains on roots compared with incidence in agar*

Inoculum = M_1 throughout; incubation time = 12 days.

	Reacting as															
	Roots								Agar							
Host	36	157	204	284	297	Un-stable	Neg.	Total	36	157	204	284	297	Un-stable	Neg.	Total
Red	6	0	2	4	6	0	2	20	0	1	0	1	9*	0	5	16
White	1	5*	1	6*	2	0	2	17	1	3*	2	5*	0	3*	5	19
Crimson	6	0	8*	0	1	2	3	20	3	0	3	2	5	0	5	18
Subterranean	4	0	0	0	7	0	6	17	3	0	2	0	3	0	10	18
Total	17	5	11	10	16	2	13	74	7	4	7	8	17	3	25	71

* Significant χ^2 (1 D.F., $P < 0{\cdot}05$). Total χ^2 for host effect: Roots (18 D.F.) = 44·5, $P < 0{\cdot}001$. Agar (18 D.F.) = 49·3, $P < 0{\cdot}001$

Influence of the host. The data have been analysed for possible host effect on the six occasions represented by inocula M_2, M_3 and M_4, each at 3 and 10 days.

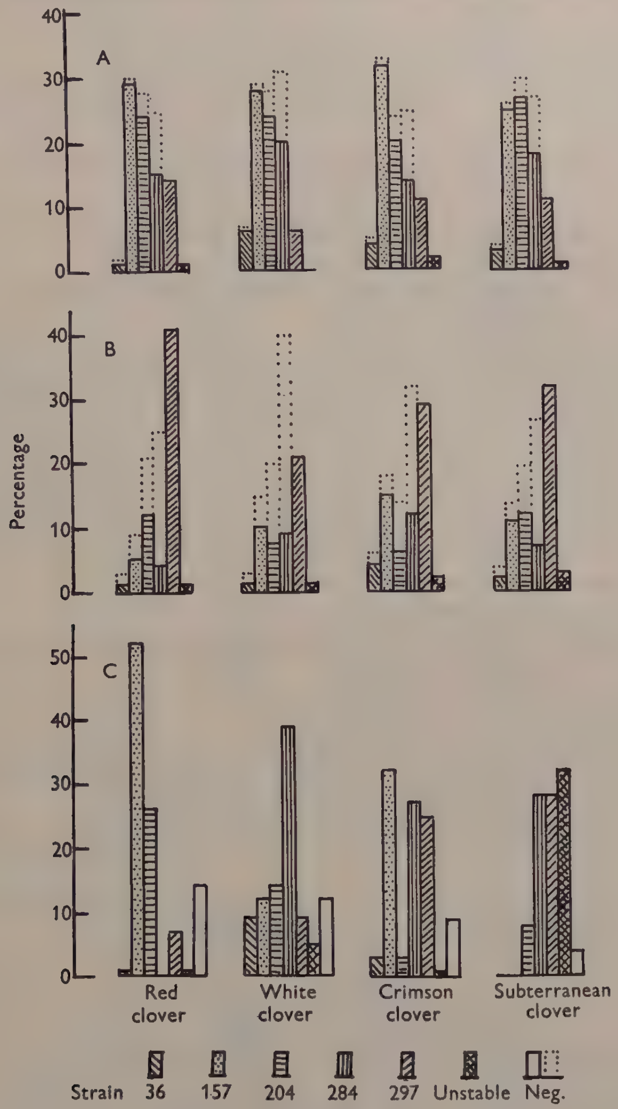


Fig. 1. Proportion of strains according to host. A, outside host, 3 days; B, outside host, 10 days; C, from nodules.

In each case the proportions of strains for each host has been compared with the average for the four hosts:

Treatment	χ^2(18 D.F.)	P
3 days, M_2	33·5	0·05–0·02
3 days, M_3	16·7	0·8–0·7
3 days, M_4	16·2	0·8–0·7
10 days, M_2	33·9	0·05–0·02
10 days, M_3	33·8	0·05–0·02
10 days, M_4	21·1	0·5–0·3

Although in three of the six cases the χ^2 test is significant, there are no marked trends due to host. (Fig. 1A, B). Noting the results in more detail:

(*a*) Red clover: deficiency of strain 284, M_3 at 10 days.

(*b*) White clover: no significant excess or deficiency.

(*c*) Crimson clover: excess 36 (10 days, M_2) and 157 (10 days, M_3), each on one occasion.

(*d*) Subterranean clover: excess 204, deficiency 157, each on one occasion (3 days, M_2).

Strain 36 is generally a minor constituent, but the other four have fair representation at one stage or another.

An analysis of the frequency of occurrence of strain 157 failed to show any host effect (F, 3/24: 1·7, $P > 0·05$).

Proportion of strains in the immediate root environment. A subsidiary test (inoculum M_1, 12 days) was made to determine whether the population closest to the root showed any greater host effect than the growth elsewhere in the seedling agar (Table 4). Although in this smaller test there were significant departures from random distribution, this was no more marked in the root than in the agar population. The proportions of strains on the roots and in the agar, based on the totals for all hosts, were not different ($P = 0·2 - 0·1$). In more detail this was the result with each host:

(*a*) Red clover: excess 297 (agar).

(*b*) White clover: excess 157 and 284 (plant and agar); deficiency of strain 297 (agar).

(*c*) Crimson clover: excess 204 (plant).

(*d*) Subterranean clover: no significant excess or deficiency.

Comparison of these results with those for inocula M_2-M_4 emphasizes the absence of any trend associated with a particular host.

Strain 36 occurred in this set of tubes more frequently than with the other inocula levels.

Colonies obtained from the rhizosphere when the plants were harvested at 6 weeks also failed to show any marked host influence:

Clover host	Percentage due to strain					Unstable antigen	Neg.	Total cases
	36	157	204	284	297			
Red	9	3	18	18	28	9	15	33
White	0	15	30	15	22	0	18	27
Crimson	0	13	30	26	20	6	5	54
Subterranean	0	12	24	9	27	0	28	33
Total, 6 weeks	2	11	26	18	24	4	15	147
Total, 10 days	2	10	10	8	31	2	37	469

χ^2, 6 weeks material, distribution of strains as a function of host (18 D.F.) = 27·4 ($P = 0·10$–$0·05$). No individual cases were significant.

Stage of growth. Strains 157 and 204 were well represented in the 3-day population but were largely replaced by strain 297 at 10 days (compare

Figs. 1A and 1B, 2A and 2B). The changes in proportion of strains are highly significant for each inoculum level:

Inoculum	χ^2 (6 D.F.)	P
M_2	57·9	$<0{\cdot}001$
M_3	35·8	$<0{\cdot}001$
M_4	77·8	$<0{\cdot}001$

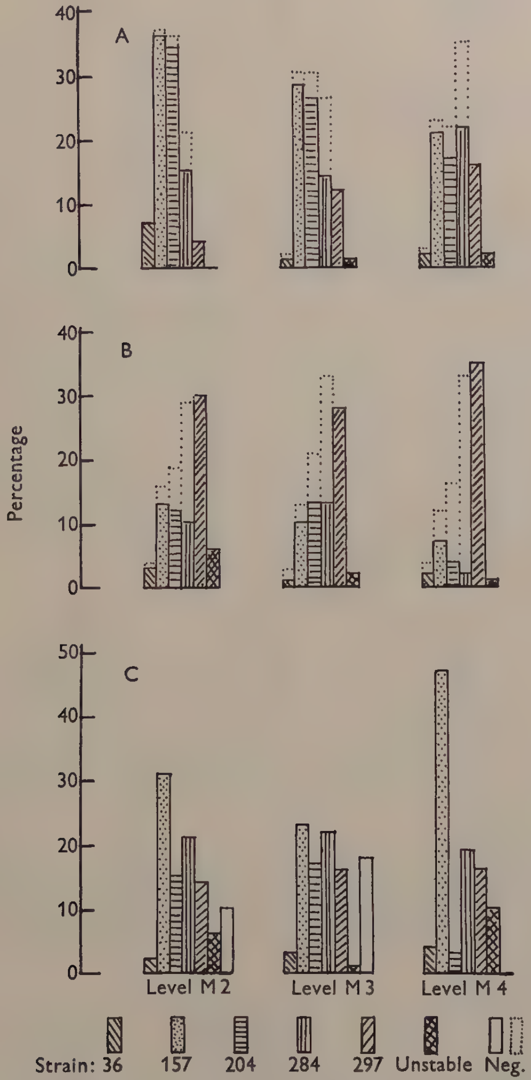


Fig. 2. Proportion of strains according to inoculum level. A, outside host, 3 days; B, outside host, 10 days; C, from nodules.

Similarly, the analysis of strain 157 showed a highly significant change in proportion of the strain with time (F, 1/24 = 35·6, $P < 0{\cdot}01$).

Comparing the 10-day with the 6-week result above, strains 204 and 284 were more common on the later occasion, negative reactors less frequent. χ^2 (6 D.F.) = 51·2, $P < 0{\cdot}001$.

Influence of inoculum level. There is a definite shift in the relative proportion of strains according to the size of the inoculum (Fig. 2A, B). This is most marked at 3 days (Fig. 2A), but can be seen in the 10-day result as well (Fig. 2B). These results are statistically highly significant:

Stage of growth	χ^2 (12 D.F.)	P
3 days	42·6	<0·001
10 days	32·6	0·01–0·001

The analysis of strain 157 showed a significant change with inoculum level (F, 2/24 = 4·8; $P = 0{\cdot}05 - 0{\cdot}01$).

Ratio of strains in the nodules

Full data for the several inocula levels are set out in Table 5. The agreement between replicate tubes is probably as good as could be expected for the necessarily small numbers per tube. A χ^2 test has been applied to the distribution of culture types for cases having replicates. In no case was there any significant difference between replicates. Total χ^2 (132 D.F.) was 120·0, $P > 0{\cdot}9$. The standard deviation of strain 157, based on the replicate tubes in the M$_2$ data was ±1·4. Totals of replicates are utilized in subsequent comparisons.

Influence of the host. Percentages for the separate hosts (inoculum levels pooled) are plotted in Fig. 1C. Unlike the cultures obtained from the growth outside the root, the proportion of the several strains found in the nodules is markedly affected by the host species. Considering the distribution of strains for the four hosts at each inoculum level:

Inoculum	χ^2 (18 D.F.)	P
M$_2$	65·5	<0·001
M$_3$	36·9	0·01–0·001
M$_4$	48·7	<0·001
All (Fig. 1C)	143·4	<0·001

The analysis of 157 obtained from the plants of the M$_2$ tubes for which several replicates were available showed a highly significant host effect (F, 3/8 = 13·5, $P < 0{\cdot}01$).

Influence of inoculum level. On the other hand, the proportion of strains for the one host is largely independent of inoculum concentration. Taking the data for each host separately, there was a significant inoculum effect in the case of white clover but this is almost entirely due to the results in a single tube with inoculum M$_4$:

Clover host	χ^2 (12 D.F.)	P
Red	13·4	0·5–0·3
White	33·5	0·01
Crimson	9·0	0·7
Subterranean	15·4	0·3–0·2
All hosts (Fig. 2C)	24·7	0·05–0·02

The variant tube with white clover had a larger proportion of strain 157 and less 284 than for the three inocula generally. The results with all hosts are significant, but this is due to some excess of 157 and deficiency of 204 and non-reactors in inoculum M$_4$. No real trends can be detected in these results.

Table 5. *Proportion of strains in nodules*

Inoculum level	Clover host	Reacting as							
		36	157	204	284	297	Un-stable	Neg.	Total
M_2	Red	0	7	3	0	0	0	0	10
		0	8	1	0	0	1	0	10
		0	4	6	0	0	0	0	10
	Total	0	19	10	0	0	1	0	30
	White	1	0	0	6	0	1	1	9
		0	1	2	0	3	1	1	8
		1	0	1	4	1	0	1	8
	Total	2	1	3	10	4	2	3	25
	Crimson	0	5	0	1	3	1	0	10
		0	2	0	2	2	0	1	7
		0	2	1	1	4	0	2	10
	Total	0	9	1	4	9	1	3	27
	Subterranean	0	0	0	3	0	0	0	3
		0	0	1	2	1	2	0	6
		0	0	0	2	0	1	0	3
	Total	0	0	1	7	1	3	0	12
	Total M_2	2	29	15	21	14	7	6	94
M_3	Red	0	3	4	0	3	0	0	10
		0	4	4	0	0	0	2	10
		0	6	1	0	2	0	0	9
	Total	0	13	9	0	5	0	2	29
	White	0	1	0	5	0	0	0	6
		0	0	2	1	0	0	2	5
		1	0	0	5	0	0	1	7
		0	0	2	0	1	0	1	4
	Total	1	1	4	11	1	0	4	22
	Crimson	0	2	0	0	5	0	1	8
		0	4	0	4	1	0	1	10
		2	1	1	4	1	0	1	10
	Total	2	7	1	8	7	0	3	28
	Subterranean	0	0	1	0	1	1	1	4
	Total, M_3	3	21	15	19	14	1	10	83
M_4	Red	0	9	1	0	0	0	0	10
	White	2	5	1	1	0	1	0	10
	Crimson	0	4	0	3	3	0	0	10
		0	4	0	5	0	0	0	9
	Total	0	8	0	8	3	0	0	19
	Subterranean	0	0	0	0	2	3	0	5
		0	0	0	0	3	1	0	4
	Total	0	0	0	0	5	4	0	9
	Total, M_4	2	22	2	9	8	5	0	48
M_1	Red	0	6	3	0	1	0	0	10
	Subterranean	0	0	0	0	4	0	0	4
		0	0	2	3	0	0	0	5
		0	0	0	0	3	0	0	3
		1	1	1	0	0	0	4	7
		0	1	0	0	0	0	1	2
		0	0	0	0	2	0	2	4
	Total	1	2	3	3	9	0	7	25
	Total, M_1	1	8	6	3	10	0	7	35

Negative reactors again require some consideration. In cases which were first negative, duplicate cultures picked from the same plates were in many cases positive to one of the testing sera. The ratio of strains represented by these cases was practically the same as the ratio for strains positive on the first occasion. This fact, and the relatively small proportion of negative reactors amongst nodule isolates, seems to indicate that such non-reactors would not seriously interfere with the estimate of strain proportions based on the positive cases.

The saline unstable controls, so common with subterranean clover (Fig. 1 C), are more difficult. However, even if on this account subterranean clover were excluded, the selective effect of the host plant would be apparent with the other three species.

Relationship between proportion of strains outside the root and in the nodules

It is clear from the data already given that, under the conditions of this experiment, where several strains have been able to grow together in the root environment, success in forming nodules need have little to do with their relative growth outside the root. This will be seen: (i) by comparing the major influence exerted by the four hosts on the strains producing nodules (Fig. 1 C) with their insignificant effect on the population outside the plant (Fig. 1 A, B); (ii) by observing the failure of increasing inoculum concentrations to produce in nodules the same trends (decreasing 157, increasing 297) which are so marked amongst the corresponding populations outside the plant (Fig. 2 C, cf. A and B).

Moreover, examination of the relative position of each nodule on the root failed to show any relationship between this and the strain responsible for the nodule. There was, for example, no tendency for strain 157, which grew so well at first, to be relatively more represented amongst the early formed nodules, nor for strain 297, which largely dominated the late population, to be better represented amongst the younger nodules.

DISCUSSION

The performance of strains in the nodulation of each host can be set out in decreasing order:

Red clover	White clover	Crimson clover	Subterranean clover	
157 (E)	284 (E)	157 (I)	284 (E)	Major strains
204 (E)		284 (E)	297 (E)	
		297 (E)		
297 (*i*)	204 (E)	36 (E)	204 (I)	Minor strains
36 (*e*)	157 (E)	204 (I)	36 (E)	
284 (*i*)	36 (E)		157 (I)	
	297 (E)			

(Note: (E), etc., refers to known nitrogen-fixing effectiveness with the host concerned; see Table 1 for detail.)

Other data collected in this laboratory (157 > 204, 157 > 284, for red clover; 204 > 157 for subterranean clover) have been in agreement with these findings.

At first glance one might be tempted to see some significance in the general occurrence of highly effective associates amongst those best able to produce nodules competitively. Strain 157 with crimson clover is, however, a striking exception which, taken with the fact that more effective than ineffective associations were involved amongst the host + bacterium pairings, is adequate warning against any generalization in this regard from the present data.

The evidence that competitiveness in nodule formation need not have any relationship to the relative vigour of growth outside the plant fails to support the generality of the conclusions reached by Nicol & Thornton (1942) based on the few instances they were able to study with the means then at their disposal. The conclusion that the nature of the host can so clearly determine relative success in nodulation amongst strains without reference to their relative number in the plant's immediately external environment opens up the interesting question as to the stage in the invasion process at which this selectivity operates: at the point of root hair invasion or by determining survival and growth of cells that have gained entry?

The fact that more than one serological type has not been isolated from the same nodule under conditions such that different nodules on the one plant were commonly inhabited by different strains (Hughes & Vincent, 1942) is perhaps relevant. It does not, however, provide any basis for a firm decision in favour of either of the possibilities noted above.

Relative infectivity of the strain on each of the hosts, so far as it can be assessed by earliness of nodulation and the number of nodules formed, provides at the best only a partial explanation of the host specificity reported in this paper. Unpublished data, obtained by Bockman in this laboratory by testing each strain against each host in single culture, relate both criteria reasonably well to nodulation competitiveness in the case of red clover. With the other three hosts, however, the evidence is either inconclusive or contradictory.

On the practical side, knowledge of host preferences might materially assist in the better selection of inocula for field use. This could be important in competition between inoculum and an established parasitic rhizobial population. It might also provide a solution to the difficulties associated with the simultaneous sowing of inoculated seed of two or several clover species, for which it is often difficult to find the one strain equally effective in nitrogen fixation. Strains could be chosen such that those effective for a particular host would also dominate that host's nodulation. On the present data, for example, a mixed sowing of red and subterranean clovers could be inoculated with a mixture of strains 157 (for red) and 297 (for subterranean). On the other hand, the use of mixed inocula without knowledge of host preferences would be quite irrational.

The position of negative reactors remains a somewhat unsatisfactory feature of the test. Their effect on the total result can be detected and allowed for in experiments of this kind because all isolations are expected to react with one or other of the test sera. In field tests, on the other hand, where failure to react with the test sera is taken as evidence of 'native' rhizobia, the uncertainty introduced by this phenomenon is more serious. One cause of trouble, apparently due to a heat-labile 'Vi-like' antigen capable of interfering with the somatic reaction, has been detected (Vincent, 1953). This can be avoided by the use of heated antigen and does not explain the negative cases in the present tests, all of which are based on material treated in this way. It seems that despite the high degree of serological stability that has been a feature of our experience with regularly maintained stock cultures (Purchase, Vincent & Ward, 1951) experiments involving frequent single colony re-isolations, with or without intervening plant passage, give a proportion of cultures which fail to react with the homologous antiserum (i.e. that prepared against the original stock culture). The incidence of non-reacting 'variants' seems to vary with strains, as in our present experience with strain 284, but occurs commonly enough amongst others, as indicated by the fact that negative reactors have been found in four of the five strains involved in this work. The subsequent recovery of specific reacting strains from plants inoculated with a culture in a non-reacting state suggests a fairly readily reversed variation.

More work yet remains to be done on the serology of the rhizobia, but in the meanwhile the method remains undoubtedly the best experimental tool available for detailed studies of interaction between strains of rhizobia in various environments and for field studies on success with legume inoculation.

We are pleased to acknowledge the technical assistance rendered by Miss W. Fountain and the financial assistance of the University Research Fund, the Commonwealth Bank Rural Credits Fund and the Richmond River Pasture Improvement Committee.

REFERENCES

Hughes, D. Q. & Vincent, J. M. (1942). Serological studies of the root-nodule bacteria. III. Tests of neighbouring strains of the same species. *Proc. Linn. Soc. N.S.W.* **67**, 142.

Nicol, H. & Thornton, H. G. (1942). Competition between related strains of nodule bacteria and its influence on infection of the legume host. *Proc. Roy. Soc.* B, **130**, 32.

Purchase, H. F., Vincent, J. M. & Ward, L. M. (1951). Serological studies of the root-nodule bacteria. IV. Further analysis of isolates from *Trifolium* and *Medicago*. *Proc. Linn. Soc. N.S.W.* **76**, 1.

Vincent, J. M. (1953). Vi-like antigen in clover nodule bacteria. *Aust. J. Sci.* **15**, 133.

(*Received* 8 *April* 1953)

Rainbow, C. & Mitson, G. W. (1953). *J. gen. Microbiol.* 9, 371–375.

Nutritional Requirements of Acetic Acid Bacteria

By C. RAINBOW and G. W. MITSON

Department of Applied Biochemistry, University of Birmingham

SUMMARY: Seven strains of acetic acid bacteria were separated into two well-defined groups on nutritional criteria. One group grew relatively poorly in media containing amino-acids and glucose, but grew well when lactate replaced glucose. In the presence of glucose and lactate, but not of glucose alone, ammonia was utilized as the sole source of nitrogen. With one exception, this group required no added growth factors for growth in a defined medium.

The second group grew well when glucose, but not lactate, was supplied as the chief source of carbon and/or energy. Casein acid hydrolysate was a suitable source of nitrogen, but ammonia, as a sole source of nitrogen, failed to support growth even when lactate was present, except to a limited extent when the growth period was prolonged to 7 days. All members of this group required added nicotinate, pantothenate and *p*-aminobenzoate.

The nutritional requirements of species of *Acetobacter* have as yet been little studied; this paper reports a study of the nutritional requirements of seven strains of these organisms.

METHODS

Test organisms and inocula. Seven organisms were used: (1) *Acetobacter acidum-mucosum* (NCTC 6429); (2) *A. mobile* (NCTC 6428); (3) *A. suboxydans* (NCTC 7069); (4) *A. capsulatum* (NCTC 4943); (5) *A. gluconicum* (NCTC 4739); (6) *A. turbidans* (NCTC 6249); (7) *A. viscosum* (NCTC 6426).

Cultures of each were maintained on malt agar slopes and transferred fortnightly or as required. Inocula were prepared by transferring a little of the surface growth from a slope to 6 ml. of appropriate basal medium and incubating at 28° for 40–96 hr. The cells were then washed twice (centrifuge) with 6 ml. portions of sterile 0·85 % saline and finally shaken to a homogeneous suspension in a further 6 ml. portion. One drop of this suspension was used to inoculate each test.

Basal media. (*a*) For organisms (1), (2), and (3); g./100 ml. final volume: glucose, 2·0; syrupy lactic acid (A.R.), 1·0; casein acid hydrolysate ('vitamin-free') (Allen and Hanbury), 0·6; $(NH_4)_2SO_4$ (A.R.), 0·1; KH_2PO_4 (A.R.), 0·3; $MgSO_4.7H_2O$ (A.R.), 0·2; in μg./100 ml. final volume: D-biotin, 0·08; calcium D-pantothenate, 100; thiamine, 100; pyridoxin HCl, 100; nicotinic acid, 100; riboflavin, 10; *p*-aminobenzoic acid (*p*-AB), 10; trace element solution (Emery, McLeod & Robinson, 1946), 0·05 ml. Adjustment to pH 5·8 was made with KOH solution.

(*b*) For organisms (4), (5), (6) and (7), as (*a*) above, but with lactic acid omitted and 1·0 mg. adenine/100 ml. added.

Growth tests were carried out in flat-bottomed tubes (16·5–17·2 mm. internal diameter) containing 3 ml. double-strength medium + 3 ml. of solution of test substances previously adjusted to pH 5·8 when necessary. The tubes and contents were covered with loosely fitting glass caps and sterilized by autoclaving momentarily to 15 lb./sq.in. After inoculation, the tubes were incubated in a vertical position in air at 28°. The amount of growth was determined turbidimetrically by direct reading of each tube in the Spekker absorptiometer (Northam & Norris, 1951). Results are quoted as the average readings of duplicate tubes.

RESULTS

Carbon and/or energy requirements

For these tests, the concentration of casein acid hydrolysate in the basal medium was decreased to one-half (to minimize the amount of carbon in the medium available from this source), glucose and lactate were omitted and replaced by the following test substances (10 mg./ml.): D-glucose, D-fructose, D-galactose, D-mannose, L-arabinose, D-ribose, D-xylose, lactose, maltose sucrose, D-mannitol, D-sorbitol; equimolar to glucose: erythritol, glycerol, acetate, citrate, fumarate, *a*-ketoglutarate, lactate, L-malate, succinate.

The results showed that for *A. acidum-mucosum*, *A. mobile* and *A. suboxydans* (the *A. mobile* group) lactate was the best source of carbon and/or energy. The sugars and sugar alcohols promoted relatively poor growth, or no growth above that obtained in the unsupplemented medium. The *A. mobile* group failed to grow appreciably on acids except that, after prolonged incubation, *A. acidum-mucosum* and, to a smaller extent, *A. suboxydans* grew on acetate, and *A. mobile* on succinate and malate. Glycerol was a good substrate for *A. mobile* and *A. suboxydans* after an initial lag.

The metabolism of lactate in the absence of glucose by organisms of the *A. mobile* group involved an increase in pH to 7·0–7·6 after 7 days. In contrast, glucose was metabolized by all the organisms examined (except *A. acidum-mucosum*) to acid end products (pH values of *c.* 3·0 after 7 days), probably gluconic, 2-ketogluconic and 5-ketogluconic acids, which are known to be products of glucose oxidation by *Acetobacter* species.

A. capsulatum, *A. gluconicum*, *A. turbidans* and *A. viscosum* (the *A. viscosum* group) possessed points of resemblance among themselves, whilst differing from the *A. mobile* group. These organisms failed to grow on lactate and other organic acids but grew well on glucose, mannitol and (except *A. gluconicum*), sorbitol, erythritol or glycerol. Fructose was a less effective substrate than glucose. *A. capsulatum* proved unique in growing well on maltose.

Nitrogen requirements

Casein acid hydrolysate and $(NH_4)_2SO_4$ were tested as sources of nitrogen for growth in basal media from which these substrates had been omitted. In view of the marked stimulation of the growth of three strains produced by lactate, a parallel series of tests was also carried out in media containing lactate.

The results showed that casein hydrolysate was a suitable source of nitrogen for all seven organisms. In the absence of lactate, ammonia, as a sole source of nitrogen, was not utilized for growth by the *A. mobile* group. However, when lactate was present ammonia was assimilated by these organisms and growth in the presence of casein hydrolysate was greatly stimulated.

By contrast, the *A. viscosum* group failed to grow, except to a limited extent after a prolonged period of incubation, when ammonia was provided as the sole source of nitrogen whether lactate was present or not. Furthermore, in a medium containing casein hydrolysate, growth was depressed by lactate, the effect being particularly marked with *A. capsulatum* and *A. gluconicum.*

Requirements for added growth factors

The tests with the *A. mobile* group were carried out in a defined medium consisting of the basal medium from which all growth factors and casein hydrolysate were omitted and in which the concentration of $(NH_4)_2SO_4$ was 0·3 g./100 ml. The *A. viscosum* group was tested in the appropriate basal medium from which all growth factors, except adenine, were omitted. The casein acid hydrolysate used in this medium was tested separately with suitable micro-organisms to demonstrate that it was substantially free from biotin, thiamine, pyridoxin and riboflavin; the present tests showed it to be free from *p*-AB, pantothenate and nicotinate. The seven organisms were tested in the presence and absence of all the growth factors and in the absence of each factor singly.

The results showed that *A. acidum-mucosum* and *A. suboxydans* required no added growth factors. *A. mobile* required only *p*-AB, which it required also when L-glutamic acid replaced $(NH_4)_2SO_4$. Each organism of the *A. viscosum* group showed an absolute requirement for pantothenate and nicotinate; the requirement for *p*-AB became less marked on prolonged incubation.

Adenine was included in the test medium for the *A. viscosum* group because separate tests (Table 1), carried out with the appropriate basal medium from which adenine and *p*-AB were omitted, showed that adenine was stimulatory for *A. gluconicum*, especially during the early stages of incubation, even in the presence of *p*-AB. Under similar conditions, adenine was not stimulatory for the other organisms of the *A. viscosum* group. Adenine did not substitute for *p*-AB, or did so only partially after prolonged incubation, for the organisms of

Table 1. *Adenine and* p-*AB as growth factors for acetic acid bacteria*

Tests in basal medium less adenine and *p*-AB. Age of inocula, 45 hr., except *A. gluconicum*, 96 hr. Concentrations for tests: *p*-AB, 0·1 μg./ml.; adenine, 10 μg./ml. Growth as Spekker readings.

	Incubation time (hr.)	Growth in basal medium plus			
		No additions	*p*-AB	Adenine	*p*-AB + adenine
A. capsulatum	44	0·03	0·71	0·05	0·75
	117	0·03	1·03	0·22	1·07
A. gluconicum	72	0·03	0·05	0·11	0·26
	117	0·03	0·51	0·51	0·74
A. turbidans	44	0·05	0·35	0·17	0·37
	92	0·17	0·72	0·35	0·73
A. viscosum	44	0·05	0·25	0·12	0·28
	117	0·16	0·63	0·38	0·64

this group, although it is known to do so for the growth of some organisms in media containing mixed amino acids (Lampen, Jones & Roepke, 1949; Cutts & Rainbow, 1950).

Growth of the A. mobile *group on organic acids in defined medium*

Results have been presented which show the effect on the growth of the *A. mobile* group of (*a*) organic acids in a medium containing carbon fragments supplied as acid hydrolysed casein; (*b*) lactate in media containing glucose as a potential energy source. The capacity of organic acids to support the growth of the *A. mobile* group was also tested in a defined medium containing ammonium salts as sole source of nitrogen, both in the presence and absence of glucose. The results (Table 2) showed that lactate was both a source of energy and of carbon fragments for *A. acidum-mucosum* and *A. suboxydans*, but that *A. mobile* required the presence of glucose for good growth on the lactate ammonia medium. When the incubation period was prolonged for more than 96 hr., *A. suboxydans* grew on succinate and (less well) on citrate and aconitate, provided glucose was also present. In these cases, where both glucose and organic acid were necessary, the latter may provide carbon fragments for biosynthesis, glucose providing the necessary energy. The results also showed that, when ammonium salts were the sole source of nitrogen, the *A. mobile* group failed to grow appreciably when glucose was the sole source of carbon and energy.

Table 2. *Growth of the* Acetobacter mobile *group in defined medium*

Series A: in basal medium from which casein acid hydrolysate, glucose and lactate were omitted. Concentration of $(NH_4)_2SO_4$ increased to 0·25 g./100 ml.

Series B: as series A, but containing 0·5 % of glucose in the medium.

Concentrations of test substrates: glucose, 10 mg./ml., organic acids equimolar to glucose.

Growth recorded as Spekker readings after 7 days incubation.

	Series A			Series B		
Substrate	*A. acidum -mucosum*	*A. mobile*	*A. sub-oxydans*	*A. acidum -mucosum*	*A. mobile*	*A. sub-oxydans*
None	0·01	0·01	0·01	0·03	0·05	0·03
Glucose	0·03	0·04	0·05	—	—	—
Acetate	0·01	0·02	0·01	0·03	0·10	0·09
Aconitate	0·02	0·04	0·09	0·06	0·11	0·24
Citrate	0·01	0·01	0·11	0·03	0·07	0·45
Fumarate	0·03	0·02	0·02	0·04	0·08	0·07
α-Ketoglutarate	0·06	0·02	0·07	0·04	0·10	0·11
Lactate	1·03	0·17	0·84	0·98	0·89	1·20
L-Malate	0·04	0·03	0·03	0·05	0·12	0·12
Succinate	0·04	0·02	0·02	0·06	0·11	0·61

DISCUSSION

The organisms studied fall into two groups, each with a distinct nutritional pattern. The ability to utilize ammonia as sole source of nitrogen is of interest in connexion with the proposal of this criterion for the identification of acetic acid bacteria (Vaughn, 1942). Of the organisms discussed in this paper, the *A. mobile* group possessed this ability only when lactate was present. The composition of the medium for identification tests based on the criterion suggested by Vaughn is therefore important.

The periods of induction necessary before the metabolism of acetate (by *A. acidum-mucosum* and *A. suboxydans*) malate and succinate (by *A. mobile*) and glycerol (by *A. mobile* and *A. suboxydans*) suggests that adaptive enzymes are involved. It is noteworthy that the utilization of acetate for growth by *A. acidum-mucosum* and *A. suboxydans* only took place in the presence of amino-acids.

Foda & Vaughn (1953) found that *A. melanogenum*, *A. oxydans* and *A. rancens* require added *p*-AB, nicotinate, pantothenate and certain amino-acids. The resemblance between these results and our own suggests that this particular pattern of nutritional requirements is common to all the organisms concerned and may be, as Foda & Vaughn suggested, of taxonomic value.

Although *A. gluconicum* grew well in 48 hr. on malt agar slants, it grew considerably slower on the basal medium than any other strain used in this work. It seems likely, therefore, that the basal medium lacked a factor or factors stimulatory to this organism.

REFERENCES

CUTTS, N. S. & RAINBOW, C. (1950). Studies of a yeast exacting towards *p*-aminobenzoic acid. *J. gen. Microbiol.* **4**, 150.

EMERY, W. B., MCLEOD, N. & ROBINSON, F. A. (1946). Comparative microbiological assay of members of the vitamin B complex in yeast and liver extracts. *Biochem. J.* **40**, 426.

FODA, I. O. & VAUGHN, R. H. (1953). The nutritional requirements of *Acetobacter melanogenum* and related species. *J. Bact.* **65**, 79.

LAMPEN, J. O., JONES, M. J. & ROEPKE, R. R. (1949). Mutant strains of *Escherichia coli* unable to synthesize *p*-aminobenzoic acid. *J. biol. Chem.* **180**, 423.

NORTHAM, B. E. & NORRIS, F. W. (1951). Growth requirements of *Schizosaccharomyces octosporus*, a yeast exacting towards adenine. *J. gen. Microbiol.* **5**, 502.

VAUGHN, R. H. (1942). The acetic acid bacteria. *Wallerstein Labs. Commun.* **5**, 5.

(*Received* 21 *April* 1953)

Adam, K. M. G. (1953). *J. gen. Microbiol.* 9, 376–384.

In vivo Observations on the Ciliate Protozoa inhabiting the Large Intestine of the Horse

By KATHERINE M. G. ADAM

*Department of Biology, Royal (Dick) Veterinary College, Edinburgh**

SUMMARY: The ciliate population of the large intestine of the horse shows large, daily variations. The ventral colon is the site where the ciliate fauna varies most. Two species, *Cycloposthium edentatum* and *C. dentiferum*, became established in the large intestine after passage through the stomach and small intestine. The introduction of new species into the ventral colon caused significant changes in the fauna of that part of the gut.

In a previous paper (Adam, 1951) the author reported the number and species distribution of ciliates in samples obtained from the large intestine of slaughtered horses. Since then, Alexander (1952) has devised a method of making fistulae into the large intestine of the horse, and it has therefore been possible to investigate the ciliate protozoa *in vivo*. The object of the present work was threefold: (*a*) to follow the day-to-day variations of the ciliate population when feeding a standard diet to the horse; (*b*) to study the effect of changes in the diet; (*c*) to obtain information on how the horse may acquire its ciliate fauna.

METHODS

Observations were made on two ponies designated *A* and *B*. Pony *A* had fistulae into the caecum and ventral colon and pony *B* had fistulae into the caecum, ventral and dorsal colon. Approximately 100 ml. of gut contents were taken daily from each fistula for total and differential counts of ciliates.

The counting method previously described (Adam, 1951) was slightly modified. Since the gut contents taken from the fistula were always fluid, it was more convenient to take a measured volume instead of a particular weight of the digesta. The sample, which contained suspended material, was immediately transferred to a measuring cylinder, made up to twice its volume with 5%(w/v) formalin, and allowed to stand for 15 min. After tilting the cylinder six times to mix the suspension, 100 ml. was decanted into a large flask and the volume made up to 1, 2 or more litres with tap water, depending on the dilution required to give a suitable concentration for counting. To 2 ml. of this suspension were added 2 drops of acid methyl green and 2 ml. of glycerol, and counts of ciliates were done in a Sedgwick-Rafter cell of 1 ml. capacity. This simplified method of estimating the total number of ciliates in a sample of gut contents is both rapid and accurate. Duplicate counts made on the digesta at the various stages of dilution did not differ significantly.

---

* Now at the Department of Zoology, Edinburgh University.

Differential counts were made on samples that were fixed in formalin but not diluted further. Five ml. of the concentrated suspension were placed in a test tube and a few drops of acid methyl green were added to it. The suspension was well shaken before removing a drop for examination under the 4 mm. objective of the microscope. The first 200 (approximately) ciliates seen were separated into species and the proportions expressed as a percentage of the total count. The accuracy of this method was tested by performing counts of 200 and 1000 ciliates in a sample which contained twelve species. Ten consecutive counts were made for each group and the mean percentage value for each species was calculated. Since the differences between the means fell within the random sampling error of the counts, it was concluded that counts of more than 200 ciliates were unnecessary.

The first series of experiments was carried out on pony *A* which was given four different diets in the following order: (1) grass only; (2) grass plus 2 lb. oats daily; (3) hay only; (4) hay plus 2 lb. oats daily. It was maintained on each diet for 3 weeks, and twenty daily records of the ciliate population were obtained for each of the four diets. The second series of experiments was carried out on pony *B*, which was fed with grass only for a period of 4 weeks and then with hay only for 4 weeks; again twenty records of the ciliate population were made for each diet.

The pH value of the gut fluid was measured with a glass electrode connected to a Marconi pH meter.

Since the composition of the ciliate population differed in the two ponies, attempts were made in various ways to introduce species from one animal into another. About 100 ml. of strained contents from the ventral colon of *A* were given to pony *B* by stomach tube. A small quantity of faecal material from pony *A* was moistened with water, mixed with oats and fed to pony *B*, and similarly from *B* to *A*; this was done on three consecutive days. Finally, species from *A*, which had not become established in the ventral colon of pony *B* by either of these methods, were introduced directly through the fistula. During the whole of this part of the experiment both ponies were kept on a hay diet.

RESULTS

Effect of diet on the concentration of ciliates

The effect of diet on the number of ciliates is shown in Table 1. The diets were fed in the order shown but, owing to the wide daily variation of the counts, it was not possible to distinguish a gradual increase or decrease in the numbers with the passage of time. This variability of the counts could not be accounted for wholly by errors in the method of counting or in sampling the fluid from the colon. The volume of sediment in the sample varied from day to day but bore no relation to the concentration of ciliates in the sample.

The difference between the means of successive pairs of results was therefore tested for significance. Thus in the caecum of pony *A* there was no detectable effect until oats were added to hay when the mean count fell ($P < 0{\cdot}001$). In the ventral colon where the ciliates were more concentrated the count rose significantly after the addition of oats to grass ($P < 0{\cdot}001$), and it again rose when the diet consisted of hay only ($P < 0{\cdot}01$). In pony *B*, the change from grass to hay caused a doubtful fall ($P < 0{\cdot}05$) in the concentration of ciliates in the ventral colon and had no effect in the caecum or dorsal colon.

Table 1. *The effect of diet on the concentration of ciliates*

Pony	Diet	Ciliates /ml.; mean* $\times 10^{-3}$	Ciliates /ml.; range $\times 10^{-3}$	Standard error of the mean	Difference between means $\times 10^{3}$	Value of P
			Caecum			
A	Grass	20·6	9–47	2·302	−3·3	<0·2
A	Grass + oats	17·3	4–36	1·936	0·0	·
A	Hay	17·3	7–33	1·549	−9·6	<0·001
A	Hay + oats	7·7	1–33	1·717		
B	Grass	13·4	3–53	2·501	−3·1	<0·9
B	Hay	10·3	6–19	0·706		
			Ventral colon			
A	Grass	111	22–162	8·672	+183	<0·001
A	Grass + oats	294	14–472	21·096	+576	<0·01
A	Hay	870	85–3072	215·385	−175	<0·5
A	Hay + oats	695	132–1928	121·944		
B	Grass	62·8	2–253	16·363	−31·4	<0·05
B	Hay	31·4	7–89	1·162		
			Dorsal colon			
B	Grass	645	67–1055	65·410	−150	<0·1
B	Hay	495	129–929	51·943		

* Each value is the mean of twenty observations.

The density of the ciliate population in each part of the gut is evident: the numbers are low and relatively constant in the caecum, high and variable in the ventral colon, high but less variable in the dorsal colon. The smaller number of ciliates in the ventral colon of pony *B* was associated with differences in the type of fauna (see later).

Effect of diet on the distribution of species

Tables 2–4 show the effect of diet on the distribution of species. The wide variability in the range of the proportions of each species is a feature common to all three tables.

Caecum. Change of diet had no pronounced effect on the composition of the fauna. *Cycloposthium bipalmatum*, *Blepharocorys jubata* and *B. uncinata* are the three species typical of the caecum. In pony *A*, where only *C. bipalmatum* and *B. jubata* were present, hay appeared to favour *C. bipalmatum* and to depress *B. jubata*. In pony *B*, where the two species of Blepharocorys occurred together, *B. uncinata* was the more abundant; it showed an increase on hay while *C. bipalmatum* decreased.

Ventral colon. Twelve species occurred in the ventral colon of pony *A* but only those which formed more than 10% of the population will be considered. The species are all characteristic of the proximal fauna but their proportions in the caecum and ventral colon differ. When the grass diets are compared with the hay diets, it is seen that on hay or hay and oats *Blepharoprosthium ampulla* became the dominant species and formed 63 and 68% of the

Table 2. *Species distribution in the caecum*

Species	Diet									
	Grass			Grass + oats		Hay			Hay + oats	
	Mean (%)	Range (%)	Estimate of total numbers /ml. $\times 10^{-3}$	Mean (%)	Range (%)	Mean (%)	Range (%)	Estimate of total numbers /ml. $\times 10^{-3}$	Mean (%)	Range (%)
Pony *A*										
Cycloposthium bipalmatum	30·0	7–48	6·2	21·7	6–40	44·6	17–86	7·8	46·7	12–92
C. dentiferum	+	.	.	+	.	0·1	0–2	.	1·0	0–4
C. edentatum	+	.	.	+	.	0·3	0–3	.	4·9	0–18
Blepharocorys jubata	36·0	1–77	7·4	43·7	6–78	23·8	5–52	4·1	16·9	0·7–62
Blepharoprosthium pireum	+	.	.	+	.	+	.	.	+	.
B. ampulla	15·3	0·4–40	3·1	19·2	9–41	18·0	1–38	3·1	10·1	1–39
Blepharozoum trizonum	7·2	0–31	1·4	5·7	0–17	6·0	0·4–21	1·0	8·8	0·8–27
Paraisotricha minuta	0·1	0–1	.	0·3	0–2	0·7	0–6	.	1·3	0–9
P. colpoidea	6·8	0·5–20	1·4	6·1	0–18	3·5	0–13	0·5	5·1	0–19
Didesmis ovalis	2·9	0–12	.	0·8	0–2	0·8	0–7	.	0·8	0–5
D. quadrata	1·0	0–5	.	1·4	0–4	1·6	0–11	.	3·8	0–36
Pony *B*										
C. bipalmatum	41	13–65	5·5	.	.	25·5	15–51	2·6	.	.
Blepharocorys uncinata	39	21–70	5·2	.	.	60·0	36–75	6·2	.	.
B. jubata	8	1–34	1·1	.	.	8·5	0–17	0·9	.	.
Blepharoprosthium ampulla	11	0–41	1·5	.	.	5·8	0–11	0·6	.	.

Table 3. *Species distribution in the ventral colon*

Species	Diet									
	Grass			Grass + oats		Hay			Hay + oats	
	Mean (%)	Range (%)	Estimate of total numbers /ml. $\times 10^{-3}$	Mean (%)	Range (%)	Mean (%)	Range (%)	Estimate of total numbers /ml. $\times 10^{-3}$	Mean (%)	Range (%)
Pony *A*										
Cycloposthium bipalmatum	0·7	0–2	0·8	0·7	0–2	0·2	0–1	1·7	0·2	0–1
C. dentiferum	0·5	0–5	0·5	0·3	0–5	0·2	0–2	1·7	0·2	0–1
C. edentatum	0·7	0–5	0·8	0·3	0–3	0·3	0–3	2·6	0·5	0–4
Blepharocorys jubata	4·3	0–11	4·4	1·6	0–5	0·7	0–4	6·1	0·3	0–2
Blepharozoum trizonum	4·1	0–16	4·4	5·3	0–13	1·8	0–12	17·4	1·6	0–7
Paraisotricha minuta	40·5	16–67	44·2	33·0	4–70	15·5	0·3–47	130·5	8·8	1–18
P. colpoidea	5·5	0–17	5·5	3·9	0–9	2·2	0·3–8	19·1	1·0	0–5
Didesmis ovalis	17·4	1–32	18·8	20·2	4–36	6·5	1–20	56·5	9·7	2–27
D. quadrata	11·0	1–31	12·2	18·0	2–37	2·5	0–16	17·4	2·6	0–15
Blepharoprosthium ampulla	13·5	3–29	14·4	13·5	1–65	63·4	5–89	551·6	67·8	32–90
B. pireum	0·9	0–4	1·0	1·0	0–4	0·8	0–2	6·9	0·5	0–2
Bundleia inflata	0·4	0–3	0·4	1·7	0–13	5·6	0–20	48·7	6·3	0·4–22
Pony *B*										
Cycloposthium bipalmatum	78	25–99	49·0	.	.	89	78–97	27·9	.	.
Blepharocorys uncinata	12	0–55	7·5	.	.	8·3	1–19	2·6	.	.
B. jubata	3	0–16	1·9	.	.	0·7	0–3	0·2	.	.
Blepharoprosthium pireum	+	.	.	.	.	0·8	0–2	.	.	.
B. ampulla	5	0–40	3·1	.	.	0·3	0–2	0·1	.	.

population. On the grass diets, however, *Paraisotricha minuta* was the most numerous with *Didesmis ovalis* and *D. quadrata* forming an appreciable part of the fauna. A further point to be noted is that the actual concentration of *P. minuta* increased from 44,200/ml. on grass to 130,500/ml. on hay, although the proportion actually fell from 40 to 15%. The proportion and number of *Bundleia inflata* rose conspicuously on hay or hay plus oats. In pony *B* the composition of the fauna was much more restricted, only five species being present; of these *C. bipalmatum* was the most abundant.

Table 4. *Species distribution in the dorsal colon*

	Diet					
	Grass			Hay		
Species	Mean (%)	Range (%)	Estimate of total numbers/ ml. × 10^{-3}	Mean (%)	Range (%)	Estimate of total numbers/ ml. × 10^{-3}
Pony *B*						
Blepharocorys microcorys	22	2–40	142	22·6	7–33	114
B. curvigula	10	3–27	64	8·4	3–14	40
B. uncinata	1	0–15	6·4	+	.	.
B. jubata	+	.	.	+	.	.
Bundleia postciliata	25	3–37	161	26·7	9–51	134
B. inflata	3	0–7	19	1·3	0–3	5
Blepharoprosthium ampulla	20	10–33	192	19·0	5–30	94
B. pireum	+	.	.	0·4	0–2	.
Charonnautes equi	11	5–34	71	6·6	1–18	34
Triadinium caudatum	2	0–5	13	5·0	1–15	25
Tetratoxum parvum	1	0–3	6·4	1·4	0–6	5
T. unifasciculatum	+	.	.	1·2	0–5	5
Cochliatoxum periachtum	+	.	.	0·9	0–7	5
Spirodinium confusum	+	.	.	0·9	0–2	5
Blepharosphaera ellipsoidalis + *Hemiprorodon gymnoposthium*	1	0–4	6·4	2·6	0–7	14
Cycloposthium bipalmatum	+	.	.	+	.	.

Dorsal colon. The variety of species observed is typical of the distal fauna. *Blepharocorys* spp. and *Bundleia* spp. are commonly numerous in this part of the gut. The proportions of each species remained remarkably constant on the two diets. Nevertheless, on the hay diet there was a slight increase in the number of oligotrichs, namely, *Triadinium caudatum*, *Tetratoxum parvum*, *T. unifasciculatum*, *Cochliatoxum periachtum* and *Spirodinium confusum*.

Transmission of ciliates

Oral administration of ciliates from pony A to pony B. Since pony *B* would not eat fresh colon contents mixed with oats, the digesta had to be introduced by stomach tube. This was done on two occasions. After the first dose, *Cycloposthium edentatum* became established in the ventral colon, and after the second dose *C. dentiferum* became part of the same fauna. *C. edentatum* was not identified until the 8th day after the dose, when a single specimen was seen. On the 10th day several specimens were seen but the species still formed less than 1 % of the population; on the 11th day it formed 7 %; on the 12th day, 11 %; on the 13th day, 14 %, and on the 14th day, 26 %, and then increased no further.

As the concentration of *C. edentatum* increased so the digesta became noticeably greyish in colour, and when allowed to stand for a few minutes a conspicuous white layer formed at the bottom of the beaker. This white layer was due to the rapid sedimentation of the relatively heavy *C. edentatum*. On one occasion the concentration of this ciliate was so dense (143,000/ml.) that the gut contents were very pale in colour and, on settling, a layer of *C. edentatum* separated out which formed about 25 % of the volume of the sample.

The spread of *C. dentiferum* was slower and not followed in the same detail. It appeared only in small numbers 2 weeks after it had been introduced: by the end of 6 weeks it was as numerous as *C. edentatum,* and these two large species of Cycloposthium remained in about equal numbers.

No new species were transmitted by feeding oats contaminated with fresh faeces. The faeces contained species which were identified mainly as members of the distal fauna; they were non-motile but their structure appeared to be unaltered; no cysts were seen.

Administration through the fistula. When the contents of the ventral colon of pony *A* were introduced directly into the ventral colon of *B* through the fistula, the new species became established rapidly. On the 3rd day after the infection, all five new species were detected in the new host; after 5 days all were well established and formed a considerable proportion of the fauna, e.g. *P. minuta* 1 %, *D. ovalis* 27 %, *D. quadrata* 25 %, *Paraisotricha colpoidea* 4 %, and *Blepharozoum trizonum* 9 %.

In pony *B* the ventral colon contents were always rather pale, straw-coloured, but when the fauna of the two ponies became the same, the gut contents of *B* became deep, olive-brown in colour, which was the typical appearance of the ventral colon contents of *A*.

The fauna of the caecum of pony *B* was not affected by the changes in the fauna of its ventral colon, but the numbers of ciliates in the dorsal colon decreased significantly ($P < 0{\cdot}001$). The results of these experiments are presented in Table 5.

Density of the population. After the introduction of *C. edentatum* into the ventral colon of pony *B*, there was approximately a sixfold increase in the total number of ciliates and this difference was significant ($P < 0{\cdot}001$). After the introduction of all species the total number showed a further fourfold increase, which was again significant ($P < 0{\cdot}001$). This brought the total number of the ciliate population in *B* very close to that of *A*.

Distribution of species. When *C. edentatum* alone was introduced into pony *B* it became the second most abundant species, supplanting *C. bipalmatum* despite the rise in concentration of this ciliate from 28,000/ml. to 37,000/ml. *B. ampulla,* which had previously formed only 0·3 % of the population increased to 47 %. *B. inflata* and *Blepharosphaera intestinalis* appeared for the first time in the fauna of the ventral colon; they had previously been confined to that of the dorsal colon. All species from pony *A* became successfully established in pony *B*, but the distribution of species in the two ponies remained different. *B. trizonum* became the dominant species in pony *B* and, although the proportion of *C. edentatum* fell from 31 to 9 %, its actual number increased slightly.

Table 5. *Effect of introducing new species into the ventral colon*

	Normal fauna				Effect of *C. edentatum* introduced into pony *B*		Effect of introducing all species from pony *A* into pony *B*	
	Pony *A*		Pony *B*					
Species	Mean (%)	Estimate of total numbers/ml. $\times 10^{-3}$	Mean (%)	Estimate of total numbers/ml. $\times 10^{-3}$	Mean (%)	Estimate of total numbers/ml. $\times 10^{-3}$	Mean (%)	Estimate of total numbers/ml. $\times 10^{-3}$
Cycloposthium bipalmatum	0·2	2	89	28·0	20	37	2	14·8
C. edentatum	0·3	2	.	.	30·7	56·2	9	66·8
C. dentiferum	0·2	2	.	.	.	.	8	59·4
Blepharoprosthium ampulla	63·4	551	0·3	0·1	42	76·8	14	104·0
B. pireum	0·8	7	0·8	0·2	1·9	3·5	0·7	5·2
Blepharocorys uncinata	.	.	8·3	2·6	1·8	3·3	+	.
B. jubata	0·7	6	0·7	0·2	0·1	0·2	+	.
Bundleia inflata	5·6	49	.	.	2·0	3·6	+	.
Blepharosphaera ellipsoidalis	+	.	.	.	+	.	0·3	.
Paraisotricha minuta	15·5	130	.	.	.	.	8·0	59·4
P. colpoidea	2·2	19	.	.	.	.	1·0	7·4
Didesmis ovalis	6·5	56	.	.	.	.	16·0	118·8
D. quadrata	2·5	17	.	.	.	.	12·0	89·1
Blepharozoum trizonum	1·8	16	.	.	.	.	27·0	200·6
	ciliates/ml. mean $\times 10^3$ 870; S.E. 215·38		ciliates/ml. mean $\times 10^3$ 31·4; S.E. 1·16		ciliates/ml. mean $\times 10^3$ 183; S.E. 29·98		ciliates/ml. mean $\times 10^3$ 743; S.E. 67·72	

pH values of the gut contents of pony *B* are given below. Each is the mean of five determinations:

	Caecum		Ventral colon		Dorsal colon	
Diet	Mean	Range	Mean	Range	Mean	range
Grass	6·84	(6·67–6·93)	6·45	(6·29–6·56)	6·35	(6·23–6·48)
Hay	6·95	(6·70–7·10)	6·51	(6·43–6·55)	6·29	(6·16–6·38)

Conjugation

In the course of this work thousands of ciliates have been examined but only rarely has conjugation been observed; it was restricted to members of the genus *Blepharocorys* and the allied species *Charonnautes equi*. After the introduction of colon contents from pony *A* into pony *B* by stomach tube, a few pairs of *C. bipalmatum* were seen in conjugation, and it was once observed in *C. edentatum*.

DISCUSSION

Diet influences the size and composition of the population of micro-organisms in the rumen (Elsden & Phillipson, 1948). The present attempt to study the effect of diet on the ciliate population in the large intestine of the horse has yielded inconclusive results. In pony *A* the concentration of ciliates in the ventral colon increased significantly when hay was fed instead of grass, and this was accompanied by definite changes in the proportions of species. In pony *B*, however, the concentration of ciliates in the ventral colon fell when hay replaced grass and the proportions of species were unchanged. The effect of adding oats to grass or hay was also variable. The results suggest that the ciliate population is likely to vary more in the ventral colon than in any other part. By contrast, the caecum appears to be a relatively unfavourable site for ciliate growth, the number there being constantly low and the species few. Again, it would seem that the population of the ventral colon exercises an effect on that of the dorsal colon: in pony *B* the number of ciliates in the dorsal colon decreased after the fauna of its ventral colon had been changed in size and composition.

The response of the ciliate population to changes in its environment is partly determined by its composition. The results of the experiments on the transmission of ciliates showed how the introduction of a new species affected the existing community. On introducing one new species, *C. edentatum*, into the ventral colon of pony *B*, the total number of ciliates increased sixfold; the proportion of *B. ampulla* rose significantly; *B. inflata* and *B. ellipsoidalis* extended their range from the dorsal into the ventral colon. After the introduction of six more species there was a further increase in the total number of ciliates accompanied by changes in the proportions of the species.

The division rate of the ciliates can be high; for example, 5 days after *D. ovalis* was introduced into the gut the concentration had increased from about 1/ml. to 249,000/ml. The large and more complex *C. edentatum* also showed a high rate of multiplication when it was colonizing the gut. Thus it seems possible for replacement to keep pace with continual loss due to the posterior movement of the food mass.

When a sample of colon contents was introduced into the host by stomach tube, two species of oligotrichs became established in the ventral colon. Holotrichs were also present in the sample but failed to appear in the colon. It is possible that the holotrichs can only be transferred in the form of cysts. Yet active ciliates from the proximal fauna, including holotrichs, are always present in small numbers in the dorsal colon and have been detected, unchanged, in the faeces. Since the faeces contains predominantly species from the distal fauna, the host is more likely to acquire these than members of the proximal fauna. Evidence in support of this view may be found in the observation of Strelkow (1939) that young horses had fewer species of ciliates than older ones and that the distal fauna contained more species than the proximal.

I am much indebted to Dr F. Alexander, who operated on ponies for me and thus made it possible for me to carry out these investigations. I am also grateful to Prof. A. Robertson for facilities given me in his Department, and to the Racecourse Totalisator Charity Trust for a grant to purchase and maintain ponies. Mr J. G. Hood has given me much help in the laboratory.

REFERENCES

Adam, K. M. G. (1951). The quantity and distribution of the ciliate protozoa in the large intestine of the horse. *Parasitology*, **41**, 301.

Alexander, F. (1952). Some functions of the large intestine of the horse. *Quart. J. exp. Physiol.* **37**, 205.

Elsden, S. R. & Phillipson, A. T. (1948). Ruminant digestion. *Annu. Rev. Biochem.* **17**, 705.

Strelkow, A. (1939). Parasitic Infusoria from the intestine of Ungulata of the family Equidae. *Uchen. Zap. pedagog. inst. im. Gercena.* **17**, 262.

(*Received* 2 *May* 1953)

Rolinson, G. N. & Lumb, M. (1953). *J. gen. Microbiol.* **9**, 385–393.

The Oxidation of Lard Oil by *Penicillium chrysogenum*

By G. N. ROLINSON and M. LUMB

Research Department, Bacteriology Division, Boots Pure Drug Co. Ltd., Nottingham

SUMMARY: Under the conditions prevailing during the early stages of certain highly aerated penicillin fermentations, lard oil and oleic acid appear to be oxidized only slowly, or not at all, by suspensions of washed mycelium of *Penicillium chrysogenum*. Lard oil and oleic acid are both oxidized rapidly, however, under the conditions present at 48 hr. and after. The optimum pH for oxidation of lard oil by suspensions of washed mycelium *c.* 48 hr. old is 8·1 and for oleic acid, *c.* 6·4, but oxidation of both lard oil and oleic acid was apparent at the pH prevailing during the early stages of the fermentation (5·5–6·0).

Mycelium from shaken flask fermentations (conditions of relatively low aeration) appeared to oxidize lard oil only slowly even during the later stages of the fermentation although oleic acid, which is the principal product of hydrolysis of lard oil, was oxidized rapidly.

The results suggest that enzymes involved in the oxidation of lard oil and oleic acid may be present only in relatively low concentration in young mycelium and be synthesized as growth takes place. The ability to oxidize lard oil also appears to be favoured by conditions of high aeration during growth.

It has been shown (Rolinson & Lumb, 1953) that in highly aerated spore-inoculated penicillin fermentations, lard oil, added as a vehicle for the anti-foam agent, is utilized as a major source of carbon by *Penicillium chrysogenum*. Suspensions of washed mycelium from similar highly aerated fermentations were also capable of oxidizing lard oil rapidly when it was added as the sole substrate, but washed mycelium grown under conditions of relatively low aeration (in shaken flasks) failed to show any increase in respiration over the endogenous rate when incubated with lard oil.

The present paper is concerned with further investigations upon factors affecting this oxidation of lard oil by *P. chrysogenum*.

METHODS

Fermentation methods. Two types of fermentation equipment were used which provided widely different conditions of aeration. Conditions of high aeration were obtained in laboratory fermenters of the type described by Lumb & Fawcett (1951) containing 3 l. medium with stirring at 1000 r.p.m. and an air flow of 6 l./min. Relatively low aeration was obtained by using 75 ml. medium in 250 ml. conical Pyrex or Hysil flasks on a rotary shaker having a speed of 220 r.p.m. and a throw of 1½ in. All fermentations were carried out at 24–26°. The medium used in all the experiments contained: corn steep liquor (solids basis), 1·5 %; lactose, 3·0 %; glucose, 0·5 %; $NaNO_3$, 0·3 %; KH_2PO_4, 0·05 %; Na_2SO_4, 0·1 %; pH adjusted to 5·8. Phenylacetic acid was added to the stirred and aerated fermentations in additions of 0·05 % every

12 hr. starting at the 24th hour. In the shaken flask fermentations, a concentration of 0·25 % phenylacetic acid was included in the medium initially. The antifoam agent used was 2·5 % cetyl alcohol in lard oil, added as required. All the fermentations were inoculated with spore suspensions prepared from freeze-dried master cultures of *P. chrysogenum* W48–701.

Manometric methods. Conventional Warburg techniques were used as described by Umbreit, Burris & Stauffer (1945). Suspensions of washed mycelium were prepared by centrifuging a sample of fermentation liquor and washing 5 times with $KH_2PO_4 + NaOH$ buffer or with a solution containing only the inorganic constituents of the medium (hereafter called salts solution). All measurements of oxygen uptake were made with manometers maintained at 26°, rocked at 120 strokes/min. This rate was found adequate in previous experiments to ensure that the rate of solution of oxygen in the liquid in the manometer flasks was not a limiting factor.

RESULTS

Experiments were carried out to determine the time taken for oxidation of lard oil to be apparent when it was added to washed mycelium. The mycelium used was 50 hr. old, taken from a highly aerated fermentation. After washing and suspending in 0·025 M-phosphate buffer pH 7·5 the mycelium was incubated in Warburg manometers. A period of 20 min. was allowed for equilibration of temperature. Endogenous respiration was measured for a further 25 min., then the substrate was tipped in and readings were continued up to 50 min. The oxygen uptake increased *c.* 150 % almost immediately after the lard oil was added. Oxidation of lard oil over a longer period of time is shown in Fig. 1. Mycelium 46 hr. old from a highly aerated fermentation was washed and suspended in 0·025 M-phosphate buffer pH 7·5 as in the previous experiment. The average endogenous Q_{O_2} of the mycelium, which remained almost

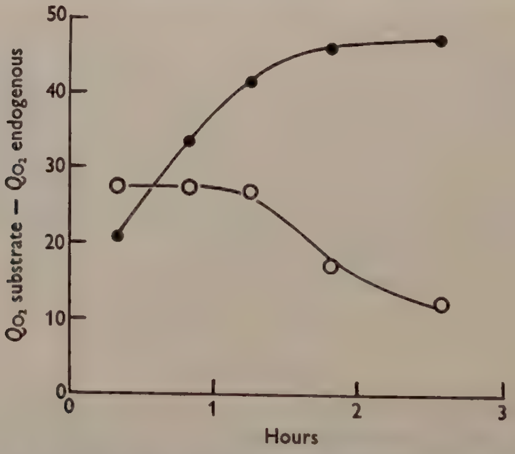


Fig. 1. Oxidation of lard oil over a prolonged period of time by washed mycelium of *P. chrysogenum*. 1 % (v/v), lard oil; ●—●; 0·1 % (v/v), lard oil, ○—○. (Endogenous Q_{O_2} = 13·1.)

constant during the whole period of the experiment, was 13·1. With 1·0 % (v/v) lard oil as sole substrate the rate of oxidation increased during the first 2 hr., although after only 15 min. the oxygen uptake was over 100 % greater than the endogenous rate. After 2 hr. the rate of oxidation was constant during the rest of the experiment. With 0·1 % (v/v) lard oil the maximum rate of oxidation was reached after only 15 min. and was maintained at a uniform rate for *c.* 75 min. Increased oxygen uptake in the presence of lard oil as substrate might be due merely to oxidation of glycerol liberated by hydrolysis (Table 1).

Table 1. *Oxidation of glycerol by* Penicillium chrysogenum

Mycelium 20 hr. old from a highly aerated fermentation, washed and suspended in 0·05 M-phosphate buffer.

Substrate	pH	Q_{O_2}	Increase over endogenous respiration (%)
Endogenous	—	22·3	—
0·05 % (v/v) glycerol	5·8	34	52·5
0·05 % (v/v) glycerol	7·0	30·3	36·0

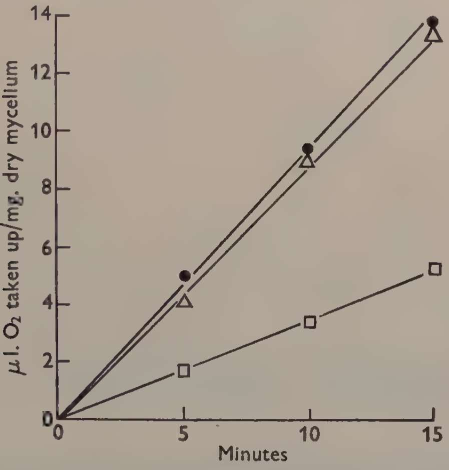


Fig. 2. Oxidation of oleic acid and sodium stearate by washed mycelium of *P. chrysogenum*. Oleic acid, 1 % (v/v), ●—●; sodium stearate, 1 % (w/v), Δ—Δ; endogenous respiration, □—□.

Oxygen uptake by washed mycelium in the presence of oleic and stearic acids, which are the chief constituent fatty acids of lard oil, is shown in Fig. 2. It will be seen that both oleic acid and sodium stearate appeared to be oxidized rapidly. The mycelium used was 69 hr. old from a highly aerated fermentation, washed and suspended in 0·05 M-phosphate buffer pH 6·4.

Effect of age of mycelium

Samples of fermentation liquor were withdrawn at different stages during a highly aerated fermentation, and the mycelium washed and suspended in the salts solution (see methods) adjusted to the pH of the fermentation at the time of sampling. The salts solution was used since it resembled the fermentation medium more closely than did the phosphate buffer. Each Warburg flask contained 1·0 ml. of the mycelial suspension and 1·0 ml. of salts solution. Oxygen uptake in the presence of lard oil and also glucose and oleic acid is shown in Fig. 3. Respiration in the absence of added substrate (endogenous respiration) was highest when the mycelium was young and declined slowly as the fermentation proceeded. With glucose as substrate (which increased oxygen uptake *c.* 85% over the endogenous rate) the maximum rate of oxidation also occurred with the young mycelium. With lard oil or oleic acid as substrate, however, the oxygen uptake of young mycelium showed no increase over the endogenous rate, and respiration was in fact slightly lower in the presence of these substrates. With mycelium 46 hr. old both lard oil and oleic acid were oxidized rapidly. With lard oil as substrate, respiration was increased over 100% and with oleic acid the increase was as much as 200% despite the relatively high endogenous rate. The maximum rate of oxidation of lard oil and oleic acid occurred with the mycelium *c.* 48 hr. old; mycelium taken at 74 hr. showed lower rates of oxidation.

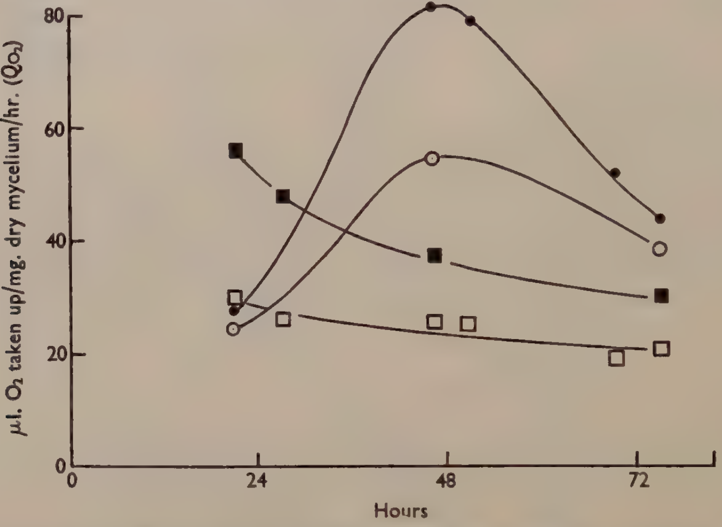


Fig. 3. Enzyme activities of washed mycelium of *P. chrysogenum* at different stages of a highly aerated fermentation. Endogenous respiration, □—□. Substrates: glucose, 1% (w/v), ■—■; oleic acid, 1% (v/v), ●—●; lard oil, 1% (v/v), ○—○.

These changes in the ability to oxidize lard oil and oleic acid might indicate the absence of the necessary enzymes in the young mycelium or might be due to unfavourable conditions for enzyme activity in the early stages of the fermentation. In the experiment shown in Fig. 3 all the samples of mycelium were suspended in the salts solution adjusted to the pH of the fermentation at that time. During the fermentation the pH rose from an initial value of *c.* 5·6 to 7·5–8·0. Thus the changes in response to lard oil and oleic acid during the fermentation might be due in part to pH.

Effect of pH *value of suspension medium*

Preliminary experiments on the effect of pH on oleic acid oxidation were carried out using mycelium washed and suspended in 0·01 M-phosphate buffers and 1·0 % (v/v) substrate. Although the buffers were too weak to maintain a constant pH value during the period of the experiment it was evident that the oxidation of oleic acid was markedly affected by the pH value, with an optimum in the region of pH 7·0. Endogenous respiration was unaffected by pH value over the range 4·3–8·3. The results shown in Fig. 4 on the effect of pH value on oleic acid oxidation were obtained using 0·1 M-phosphate buffers and 0·05 % (v/v) substrate. Under these conditions the pH remained constant during the experiment, and the relatively high concentration of phosphate did not appear to exert any unfavourable effect on respiration, although the optimum pH value under these conditions (*c.* pH 6·4) was somewhat lower than indicated from the experiments with 0·01 M buffers. Although there was a sharp optimum in the region of pH 6·4 there was measurable activity as low as pH 4·3. At pH 5·6 (pH value of the fermentation at 21 hr.) the rate of oxygen uptake was over 100 % higher than the endogenous rate.

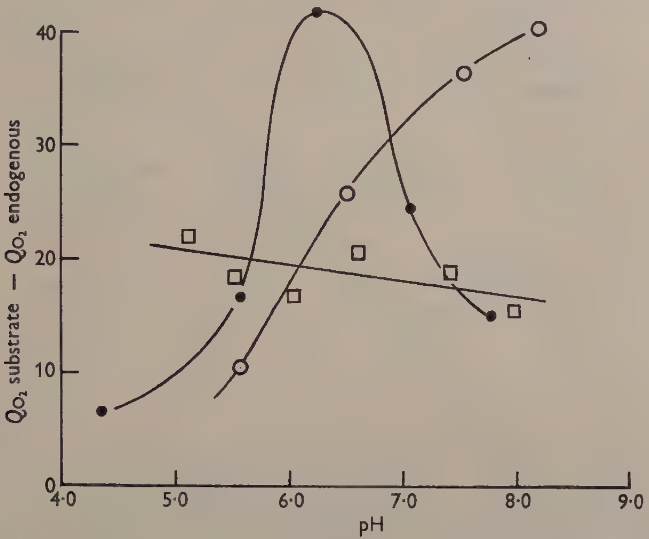


Fig. 4. Effect of pH on oxidation of lard oil, oleic acid and glucose by *P. chrysogenum* grown in a highly aerated fermentation. Glucose, 1 % (w/v), mycelium 26 hr. old, washed and suspended in 0·05 M phosphate buffers, □—□; lard oil, 1·0 % (v/v), mycelium 44 hr. old washed and suspended in 0·05 M phosphate buffers, ○—○; oleic acid, 0·05 % (v/v), mycelium 51 hr. old washed and suspended in 0·1 M-phosphate buffers, ●—●.

The experiments on pH and lard oil oxidation were carried out using 0·05 M-phosphate buffers and 1·0 % (v/v) substrate. The effect of pH was again marked. The optimum pH appeared to be > 8·1, although at pH 5·6 oxidation of the lard oil was clearly evident, the oxygen uptake being *c.* 50 % higher than the endogenous rate.

Oxidation of lard oil and oleic acid was only apparent with older mycelium. Young mycelium (*c.* 20 hr.) showed no increase in oxygen uptake in the presence of oleic acid or lard oil as substrate when suspended at the pH normally prevailing at that time (*c.* 5·6) nor when suspended at pH values near optimum for oxidation of these substrates by older mycelium. Oleic acid was added to suspensions of young mycelium adjusted to pH 6·4 and lard oil added to suspensions adjusted to pH 7·8. Both substrates were present at a concentration of 1·0% (v/v). No increase in oxygen uptake occurred over the endogenous rate, whereas at these pH values older mycelium oxidized oleic acid and lard oil rapidly.

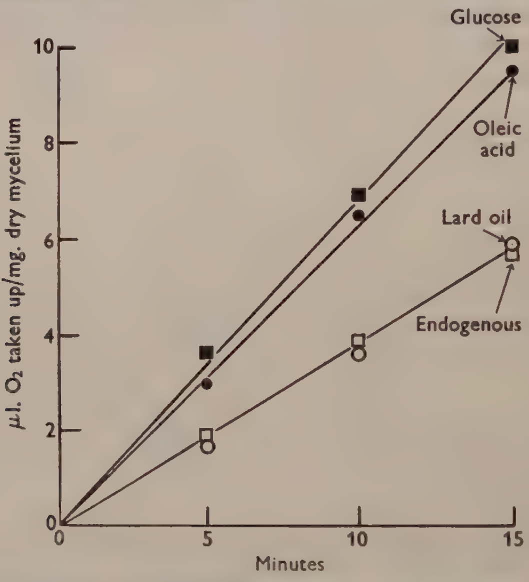


Fig. 5. Oxidation of lard oil, oleic acid and glucose by washed mycelium of *P. chrysogenum* from a shaken flask fermentation. Glucose, 1% (w/v), ■—■; oleic acid, 1% (v/v), ●—●; lard oil, 1% (v/v), ○—○. Endogenous respiration, □—□.

In contrast to the results with lard oil and oleic acid, the oxidation of glucose was little affected by pH over the range 5·1–7·95 (Fig. 4).

Effect of aeration during growth

In a previous paper (Rolinson & Lumb, 1953) it was shown that mycelium 69 hr. old, grown in a shaken flask (conditions of relatively low aeration), showed no apparent oxidation of lard oil added as sole substrate to suspensions of washed mycelium. Mycelium of similar age grown under conditions of high aeration however was able to oxidize lard oil rapidly (Fig. 3). Further experiments have shown that although the complete fat does not increase the oxygen uptake of mycelium from a shaken flask fermentation, a product of hydrolysis of lard oil such as oleic acid is oxidized rapidly (Fig. 5). In this experiment the mycelium which was 45 hr. old was washed and suspended in salts solution pH 6·9 and lard oil, oleic acid and glucose added as sole substrates. It will be seen that although the presence of lard oil did not increase the rate of respiration above the endogenous rate (despite the favourable pH for oxidation of lard oil), oxygen uptake with added oleic acid was almost as high as with glucose as substrate.

The ability of mycelium from shaken flask fermentations to oxidize oleic acid shows the same variation with age as mycelium from highly aerated fermentations. The results given in Table 2 show that whereas young mycelium showed no increase in oxygen uptake in the presence of oleic acid, with older mycelium the acid was oxidized rapidly. In this experiment the pH of both the 21 and the 69 hr. mycelial suspensions was adjusted to 6·5, which is near the optimum for oleic acid oxidation as judged from Fig. 4, yet with the young mycelium respiration with oleic acid as substrate was in fact lower than the endogenous rate.

Table 2. *Oxidation of lard oil and oleic acid by mycelium from a shaken flask fermentation*

Mycelium washed and suspended in salts solution pH 6·5.

		Q_{O_2} substrate	
Age of mycelium	Q_{O_2} endogenous	1% (v/v) lard oil	1% (v/v) oleic acid
21 hr.	12·7	13·8	7·75
69 hr.	22	20	42·0

Lard oil was not normally added to shaken flask fermentations as it was to fermentations provided with high aeration because frothing did not necessitate the use of antifoam. However, when 0·25% lard oil was included in the fermentation medium the mycelium showed no significant increase in ability to oxidize lard oil.

DISCUSSION

In the highly aerated fermentations the increase in ability to oxidize lard oil during the period 20–48 hr. corresponds with the change in the respiratory quotient (R.Q.) of such mycelium reported earlier (Rolinson, 1952). At 24 hr. the R.Q. was *c.* 1·0, but at 46 hr. it had fallen to *c.* 0·8 and then continued to fall slowly as the fermentation proceeded. Also, during the first *c.* 30 hr. of these fermentations, lactose disappeared rapidly but, after this time, this rate was only very slow (Rolinson & Lumb, 1953). The results reported here correspond with these previous results in that during the period when lactose disappearance was rapid, lard oil was oxidized only slowly or not at all, whereas at 48 hr. and after, when lactose was disappearing slowly, lard oil was oxidized rapidly.

In contrast with the changes in R.Q. during a highly aerated fermentation, the R.Q. of mycelium from shaken flask fermentations remained at *c.* 1·0 throughout the fermentation and, in the experiments described here, oxidation of lard oil by such mycelium was relatively slow during the whole fermentation.

The absence of any increase in oxygen uptake of young mycelium when lard oil was added as substrate may not indicate the complete absence of oxidation of this substrate. The endogenous respiration in these experiments was relatively high (typical of fungi). A substrate added to a suspension of washed mycelium might well be oxidized, but at the expense of substances otherwise contributing to the endogenous respiration, and thus result in no increase in overall rate of respiration. The apparent absence of lard-oil oxidation by young mycelium may only indicate a relatively lower rate of oxidation than occurs with the older mycelium.

The change in ability to oxidize lard oil and oleic acid may be due to the synthesis of the necessary enzymes during growth. Variations in enzyme activity with age have been reported for a number of bacterial enzymes (Gale, 1943) which show little or no activity in young cells but are formed during growth and reach a maximum at the time of cessation of cell division. In the experiments described here with *P. chrysogenum* the period when the ability to oxidize lard oil increased also corresponded with the period of maximum rate of growth. However, such a variation in enzyme activity might not be due to variation in enzyme content but to changes in the conditions for enzyme action such as changes in pH value of the medium or permeability of the cells during the fermentation. During the initial 24 hr. the pH value of the fermentation is not optimum for oxidation of either lard oil or oleic acid, but the results shown in Fig. 4 suggest that the pH alone would not preclude enzyme activity being apparent at this stage of the fermentation, particularly with oleic acid as substrate. However, experiments with washed mycelium suspended in buffers do not rule out the possibility that the internal pH of the mycelium is not the same as that of the surrounding medium. The presence during the early stages of growth of chemical factors which might inhibit enzyme action also cannot be overlooked, although this might be expected to be minimized by the washing procedure.

Variation in permeability of the mycelium to lard oil and oleic acid might also be responsible for variations in enzyme activity with age. However, experiments showed that no appreciable period of time after adding substrate (lard oil) was required before an increased rate of oxygen uptake was observed with 50 hr. mycelium, yet with young mycelium no increase in respiration over the endogenous rate was found over 30 min. after lard oil was added. It is also not unlikely that the whole fat does not penetrate the cell but is hydrolysed outside the cell membrane. Furthermore, if the variations in enzyme activity with age were due to changes in permeability it would also be necessary to postulate a decrease in permeability after *c.* 48 hr. when enzyme activity declined (Fig. 3).

The inability of young mycelium to oxidize lard oil is not due only to an inability to oxidize the constituent fatty acids at this stage. The fat is apparently not hydrolysed, because glycerol, which would be liberated by hydrolysis, was readily oxidized by young mycelium at pH 5·3 (Table 1).

Oxidation of oleic acid by mycelium grown under conditions of relatively low aeration showed a similar variation with age as seen with the mycelium from highly aerated fermentations. The rate of oxidation of lard oil, however, was relatively low throughout the fermentation. The level of aeration during growth thus affected the ability of the mycelium to metabolize the complete fat rather than a constituent fatty acid. Although conclusions cannot be made with certainty, it seems probable that enzymes involved in the oxidation of lard oil and oleic acid are present only in low concentration in young mycelium and are formed as further growth takes place. If this is true, the degree of aeration would appear to influence the synthesis of those enzymes involved in the hydrolysis of the fat.

The authors are indebted to the late Sir Jack Drummond, F.R.S., and to Mr C. E. Coulthard for their interest in the work, to Mr R. Fawcett for assistance in carrying out the fermentations, and to Mr C. K. Mercer and Mr J. Ransom for technical assistance.

REFERENCES

GALE, E. F. (1943). Factors influencing the enzymic activities of bacteria. *Bact. Rev.* **7**, 139.

LUMB, M. & FAWCETT, R. (1951). Improvements in experimental fermenters. *J. appl. Chem.* **1**, S 94.

ROLINSON, G. N. (1952). Respiration of *Penicillium chrysogenum* in penicillin fermentations. *J. gen. Microbiol.* **6**, 336.

ROLINSON, G. N. & LUMB, M. (1953). The effect of aeration on the utilization of respiratory substrates by *Penicillium chrysogenum* in submerged culture. *J. gen. Microbiol.* **8**, 265.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1945). *Manometric Techniques and related Methods for the Study of Tissue Metabolism.* Minneapolis, Minn.: Burgess.

(*Received* 7 *May* 1953)

WORK, E. & DEWEY, D. L. (1953). *J. gen. Microbiol.* 9, 394–409.

The Distribution of α,ε-Diaminopimelic Acid among various Micro-organisms

BY ELIZABETH WORK AND D. L. DEWEY

Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

With a Note by R. L. M. SYNGE on the 'Occurrence of Diaminopimelic Acid in some Intestinal Micro-organisms from Farm Animals'

SUMMARY: Paper chromatograms of hydrolysates of 118 micro-organisms were examined in a study of the distribution of α,ε-diaminopimelic acid and other amino-acids. A method for the identification of α,ε-diaminopimelic acid is described. Diaminopimelic acid was found in nearly all the bacteria examined, except for the Gram-positive cocci, *Streptomyces* spp., and *Actinomyces* spp. It was also found in blue-green algae but in no other algae, nor in fungi, yeasts, plant viruses, or protozoa.

Each species examined showed a different amino acid composition. β-Alanine and α- and γ-aminobutyric acids were sometimes found, often in several species of the same genus. Seven unidentified ninhydrin-reacting spots were recorded; none of them had the wide distribution of diaminopimelic acid.

During an examination of the amino acids of *Corynebacterium diphtheriae* by paper chromatography, acid hydrolysates of ethanol-washed cells were found to contain an unknown amino acid, which was subsequently isolated and identified as α,ε-diaminopimelic acid:

$$COOH.CH(NH_2).CH_2.CH_2.CH_2.CH(NH_2).COOH,$$

for brevity subsequently referred to here as diaminopimelic acid (Work, 1949; 1950*a*, *b*; 1951). At the same time, an amino acid with identical chromatographic behaviour was found in hydrolysates from antigenic lipopolysaccharides of *Mycobacterium tuberculosis*, from rumen contents of sheep, and also from soil (Asselineau, Choucroun & Lederer, 1950; Klungsøyr & Synge, footnote in Work, 1950*a*; Bremner, 1950). The isolation of diaminopimelic acid from whole *Myco. tuberculosis* (Work, 1951) confirmed the identity of the amino acid found by Asselineau *et al.* The same amino acid was also identified chromatographically in hydrolysates of *Proteus vulgaris* and *Bacterium coli* (Work, 1950*b*; Dewey & Work, 1952), *Vibrio cholerae* and numerous strains of Mycobacterium (Blass, Lecomte & Macheboeuf, 1951; Gendre & Lederer, 1952; Pauletta & Defranceschi, 1952), but it was not found in *Staphylococcus aureus* (Work, 1950*b*; Gendre & Lederer, 1952). None of these workers found diaminopimelic acid on chromatograms of products of non-bacterial origin, such as yeasts (Lindan & Work, 1951), animal fodders or animal proteins, nor has the amino acid been reported in the extensive literature on chromatography of many tissue fluids or protein hydrolysates. A derivative,

α,ϵ-diamino-β-hydroxypimelic acid was found by Woolley, Schaffner & Braun (1952) in the toxin of *Pseudomonas tabaci*.

The present investigation was undertaken to test the validity of the suggestion made by Work (1951) that diaminopimelic acid might be confined to certain bacteria. A survey of the amino acid composition of some representative bacteria, fungi, algae and other micro-organisms was therefore carried out. A preliminary report has already been given (Work & Dewey, 1952). The work has been extended by Dr R. L. M. Synge, whose quantitative examination for diaminopimelic acid in some ruminant intestinal micro-organisms is given in his note.

METHODS

Micro-organisms used. The micro-organisms were grown and harvested in the normal way for each organism, either in this Department, or by other workers to whom we are much indebted. The organisms were usually washed free of medium, but as the media were found to be free from diaminopimelic acid, washing was not essential and was omitted if not convenient. The organisms were dried, either at 100° or by acetone washing.

Examination for diaminopimelic acid. The essentials of the procedure for examining hydrolysates of micro-organisms for diaminopimelic acid have already been described (Work, 1951). Dried organisms (100–500 mg.) were hydrolysed with 10 ml. 6 N-HCl under reflux for 24 hr. HCl was removed from the hydrolysate by three evaporations to dryness *in vacuo*, and the residue was redissolved in water to final concentration 6 ml. equivalent to 1 g. dried organism. Two-dimensional chromatograms were run at 26° from 15 μl. of hydrolysate (equivalent to 2·5 mg. dry cells) applied to Whatman no. 4 paper (54 × 45 cm.). Phenol/water (NH_3+HCN atmosphere) was the first solvent, and collidine/lutidine/water the second solvent (Dent, 1948). Six chromatograms were run simultaneously from the same trough (Lindan & Work, 1951). The solvents were removed from the paper by a stream of air at 45°; spots were developed at 100° after spraying with a solution of ninhydrin in butanol (0·1 %, w/v). Since the amount of hydrolysate used for the chromatograms was based on dry weight, it was sometimes necessary, when examining organisms of abnormal protein content, to make further chromatograms using a different amount of hydrolysate in order to obtain good definition.

After the preliminary chromatograms, the remaining hydrolysate was electrodialysed to remove all basic and acidic substances. This was carried out after removal of humin by centrifugation, the hydrolysate and humin washings being diluted suitably and placed in the centre compartment (10 ml. capacity) of an electrodialysis apparatus described by Work (1950*a*), using formalin-treated parchment semipermeable membranes. A d.c. potential was applied until 1 hr. after the initial current (about 100 ma.) had fallen to a minimal value (10 ma.) and the pH value of the centre compartment had reached neutrality. The contents of the neutral centre compartment were concentrated to their original volume and chromatographed as before. If no spot was found in the position of diaminopimelic acid (spot 17, Fig. 1), the chromatograms were repeated using larger volumes (up to 200 μl.) of electrodialysed hydrolysate; if no spot then appeared the organism was said to contain no diaminopimelic acid. A spot in the correct position on any chromatogram from an electrodialysed hydrolysate was taken as evidence for diaminopimelic acid only if three conditions were fulfilled: (1) Absence of glutamic acid and aspartic acid spots, showing that electrodialysis was complete. When the condition was not fulfilled electrodialysis was repeated, to be sure that ethanolamine-*o*-phosphoric acid was removed. (2) Presence of the spot on chromatograms run from electrodialysed hydrolysate treated with 20 μl. H_2O_2 (100 vol.) + 2·5 μl. ammonium molybdate (0·4 %, w/v), thus distinguishing it from cystine (Dent, 1948). (3) Exact matching of the position of the spot with that produced by an authentic sample of diaminopimelic acid chromatographed under identical conditions.

Rough assessments of diaminopimelic acid concentration were made by visual comparison of the size and colour strength of spots given by known amounts of the amino-acid, with the size and strength of the spot in chromatograms of the electrodialysed hydrolysed micro-organisms. All other spots on the chromatograms were recorded; any unidentified spots were noted but not usually further investigated.

Preparation of fractions from organisms. Ethanolic extracts were made by extracting the wet organisms three times for 24 hr. each with 10 vol. of 70 % (w/v) aqueous ethanol; the combined extract was shaken with 3 vol. chloroform and the aqueous supernatant phase used for chromatography (Lindan & Work, 1951). *Bact. coli* soluble protein fraction was prepared by grinding acetone-dried cells as a paste with 'Filter-cel' (Johns-Manville) and 0·1 M-phosphate buffer (pH 6·8) in the ratio cells/Filter-cel/buffer/1 : 2 : 6. The mixture was centrifuged at 25,000*g* for 40 min. and the supernatant solution dialysed against water.

C. diphtheriae protein fractions were prepared by extracting acetone-dried cells (45 g.) for 2 days at +2° with 2200 ml. 2 % (w/v) ammonium sulphate solution. The mixture was filtered, the filtrate brought to 0·8 % saturation with ammonium sulphate and the resulting precipitate collected by filtration. The filtrate was saturated with ammonium sulphate and the resulting precipitate collected. The material precipitated at 0·8 % saturation was dissolved in water and further fractionated with ammonium sulphate, precipitates being collected at 0·5 and 0·8 % saturation. The three precipitates were dissolved in water and dialysed. All these procedures were carried out at +2°.

RESULTS

Chromatography of diaminopimelic acid

The behaviour of diaminopimelic acid on paper chromatograms with various solvent systems is shown in Table 1, with figures for neighbouring amino-acids included for reference. (R_f values in other solvents are quoted by Gendre & Lederer, 1952; and Wright & Cresson, 1953). Figs. 1 and 2 are typical chromatograms of a bacterial hydrolysate before and after electrodialysis. Table 1 and Figs. 1 and 2 show that the R_f value of an amino-acid is by no means an absolute constant but varies with the pH of the solvent system and with the composition of the mixture being chromatographed (see Landua, Fuerst & Awapara, 1951). The R_f value of the diaminopimelic acid spot is particularly sensitive to pH, varying from 0·18 when the acidic whole hydrolysate is chromatographed, to 0·33 when the neutral electrodialysate is examined.

Table 1. *Chromatographic behaviour in various aqueous solvent systems of diaminopimelic acid compared with neighbouring amino acids*

Except where stated, amino acids were applied to chromatograms as hydrochlorides in dilute HCl.

Solvent system ...	Phenol,* NH_3 atmos.	Phenol,* acetic atmos.	Collidine*/ lutidine	Butanol†/ acetic acid	Ethanol‡/ ammonia	Pyridine§/ amyl alcohol	Cresol
Characteristic recorded ...	R_f	R_f	R gly.‖	R gly.‖	R_f	R gly.‖	R_f
Diaminopimelic acid	0·33 ¶ 0·25	0·14	0·22	0·17	0·14	0·37	0·02
Aspartic acid	0·19	0·21	0·5	0·53	0·20	0·38	0·02
Glutamic acid	0·28	0·38	0·62	1·0	0·27	0·5	0·09
Glycine	0·44	0·41	1·0	1·0	0·46	1·0	0·14
Cystine	0·25	0·22	0·3	0·17	0·24	0·46	0·04
Ethanolamine-*o*-phosphoric acid	0·26	0·39	0·22	0·23	0·36	0·36	0·09

* Dent, 1948.

† Campbell, Work & Mellanby, 1951.

‡ Ethanol 77: 2N ammonia 33.

§ Pyridine 35: amyl alcohol 35: water 30. Edman, 1945.

‖ Ratio: $\frac{\text{distance run by amino acid}}{\text{distance run by glycine}}$.

¶ Free amino acid.

The minimum detectable concentration of diaminopimelic acid on a two-dimensional phenol/collidine chromatogram was 2 μg.; on one-dimensional chromatograms in phenol it was as low as 0·5 μg. During electrodialysis, varying proportions (up to 50%) of diaminopimelic acid passed to the cathode with the other neutral amino-acids. Therefore, when no diaminopimelic acid spot was apparent on chromatograms from 200 μl. of electrodialysed hydrolysate (= 33 mg. dry cells), the diaminopimelic acid content of the cells must have been less than *c.* 0·02%.

Absence of diaminopimelic acid from growth media

Hydrolysates of peptone, casein, yeast extract, blood, Lab Lemco, gelatin and agar-agar were all found to be free from diaminopimelic acid. It is thus evident that the usual growth media do not contain diaminopimelic acid.

Chromatography of whole micro-organisms

Table 2 shows the results obtained from the examination of chromatograms of hydrolysed micro-organisms. The approximate amount of diaminopimelic acid found in each organism is given; in certain cases (marked by §) the findings were checked by the use of an enzyme, found in the coli-aerogenes group of bacteria, which specifically decarboxylates diaminopimelic acid

Table 2. *Diaminopimelic acid (DAP) and other amino acids not common to protein hydrolysates, observed in chromatograms of hydrolysed micro-organisms examined both before and after electrodialysis*

Only one strain of each organism was examined, unless otherwise indicated by a number in brackets after the specific name.

Family	Organism	DAP content*	Other spots†	Source of cultures examined‡
	Gram-negative EUBACTERIALES			
Nitrobacteriaceae	*Thiobacillus denitrificans*	+	G	23
Pseudomonadaceae	*Pseudomonas aeruginosa*	+	–	27
	Acetobacter xylinum	+	13	27
	A. mobile	tr.	–	27
	Vibrio cholerae, Ogawa	++	16, D	1
	V. comma, El Tor	+	16	1
	Desulphovibrio desulphuricans	+	–	18
Azotobacteriaceae	*Azotobacter chroococcum*	+	–	23
Rhizobiaceae	*Rhizobium* sp.	+	–	29
Neisseriaceae	*Neisseria catarrhalis*	+	–	30
Enterobacteriaceae	*Bacterium coli*, type 1 (7)	+	–	1, 26, 30
	Aerobacter aerogenes	+	–	27
	Klebsiella pneumoniae (2)	+	D (both)	28, 30
	Proteus vulgaris ‖ (2)	+, tr.	–	25, 27
	Salmonella typhi (4)	+	16 (all) D (3 str.)	1
	Salmonella typhimurium	++§	–	1
	Shigella dysenteriae (2, rough and smooth)	+	–	13
	S. paradysenteriae	+	14	1
Parvobacteriaceae	*Pasteurella pestis*, virulent (3)	++, tr.	16 (all)	13
	P. pestis, avirulent (3)	+, tr.	16 (all) 14 (1 str.)	13
	Brucella abortus, virulent	++§	16	13
	B. abortus, avirulent	+	–	11
	Haemophilus bronchisepticus	tr.	–	20
	H. pertussis	tr.	–	20
	Gram-positive EUBACTERIALES			
Micrococcaceae	*Staphylococcus aureus*‖ (*Micrococcus pyogenes* var. *aureus*)	0	–	1, 25
	Micrococcus lysodeikticus	0	–	27
	Sarcina lutea	0	–	27
Lactobacteriaceae	*Streptococcus pneumoniae*	0	–	20
	Strep. pyogenes	0	–	1
	Strep. faecalis (2)	0	–	27
	Leuconostoc mesenteroides	0	–	27
	Lactobacillus plantarum (*arabinosus*)	++	13	27
	Propionibacterium rubrum	++	B	9
	P. thoenii	++	–	9
	P. zeae	++	–	9
	P. peterssonii	++	13	9
	P. jensenii	++	13, 19	9
	P. arabinosum	++	13, B	9
	P. pentosaceum	++	13, B, 19	9

Table 2 (*cont.*)

Family	Organism	DAP content*	Other spots†	Source of cultures examined‡
	Gram-positive Eubacteriales (*cont.*)			
Corynebacteriaceae	*Corynebacterium diphtheriae* (2)‖	++§	–	14
Bacillaceae	*Bacillus subtilis*	+	–	27
	B. brevis	tr.	–	17
	B. pumilus	+	F, 13	8
	Clostridium novyi (*oedematiens*)	+	–	20
	Cl. perfringens (*welchii*) (2)	++	13 (1 str.)	14
	Cl. tetani	0	13	14
	Rhodobacteriineae			
Athiorhodaceae	*Rhodopseudomonas palustris* (2)	++§	–	21, 23
	R. spheroides (2)	++	13 (both) 14 (1 str.)	23
	Rhodopseudomonas sp.	++	–	24
	Rhodospirillum rubrum	+	–	23
	Actinomycetales			
Mycobacteriaceae	*Mycobacterium tuberculosis* var. *hominis*‖	++	–	11
	Myco. tuberculosis var. *bovis*‖	++	19	11
	Myco. tuberculosis var. *bovis* (BCG)‖	++	19	11
	Myco. avium‖	++	19	11
Actinomycetaceae	*Actinomyces* spp.	0	A	Local
Streptomycetaceae	*Streptomyces* spp. (3)	0§	A (all)	6
	Myxobacteriales			
Cytophagaceae	*Cytophaga globulosa*	+	–	23
	Cytophaga sp. (marine)	+	–	23
	Algae			
Myxophyceae	*Anabaena cylindrica*	+	D	10
	Oscillatoria sp.	+	–	10
	Mastigocladus laminosus	+	–	10
Xanthophyceae	*Tribonema aequale*	0	–	10
Chlorophyceae	*Chlorella pyrenoidosa*	0	–	10
	Acetabularia mediterraneae	0	–	5
Bacillariophyceae	*Navicula pelliculosa*	0	–	10
Euglenineae	*Euglena gracilis*	0	–	10
Phaeophyceae	*Fucus serratus*	0	–	3
Rhodophyceae	*Rhodymenia palmata*	0	–	3
Unclassified	'Acid algae' (Allen, 1952)	0	–	23
	Yeasts			
	Brewer's yeast¶	0	–	Commer.
	Baker's yeast¶	0	–	Commer.
	Saccharomyces fragilis	0	–	7
	Fungi			
Ascomycetes	*Neurospora crassa*	0	19	15
Fungi Imperfecti	*Aspergillus flavus-oryzae*	0	19	4
	A. glaucus	0	19	4
	A. niger	0	–	4

Table 2 (*cont.*)

Family	Organism	DAP content*	Other spots†	Source of cultures examined‡
	FUNGI (*cont.*)			
Fungi Imperfecti	*A. oryzae*	0	19	4
	A. ruber	0	19	4
	Penicillium cyclopium	0	13	Local
	P. notatum	0	—	4
	P. spinulosum	0	13	Local
	Scopulariopsis brevicaulis	0	—	4
	Cephalosporium acremonium	0	—	6
	Microsporum audouni	0	—	12
	M. canis	0	—	12
	M. gypseum	0	14, 19	2
	Trichophyton rubrum	0	—	12
Basidiomycetes	*Schizophyllum commune*	0	—	4
	PROTOZOA			
	Strigomonas oncopelti	0	—	19
	Tetrahymena pyriformis	0	15	19
	PLANT VIRUS			
	Tobacco mosaic	0	—	16
	Turnip yellow mosaic	0	E	16

* 0=less than 0·02% (dry wt.); tr.=up to about 0·1%; +=0·1–0·8%; ++=more than 0·8%.

† All spots not normally found in chromatograms of protein hydrolysates. See black spots, Fig. 3 for key to location.

‡ See list of acknowledgements.

§ Checked by specific decarboxylase (Dewey, 1952).

|| Work (1951). ¶ Lindan & Work (1951).

(Dewey & Work, 1952; Dewey, 1952). Any unidentified spots found on the chromatograms are also recorded, a key to their position being given in Fig. 3. Also included in Fig. 3 and Table 2 are α- and γ-aminobutyric acids and β-alanine. These amino-acids are not normally found in protein hydrolysates but are known to be present in non-protein fractions of some micro-organisms (Work, 1949; Lindan & Work, 1951; Fowden, 1951; Blass *et al.* 1951; Pauletta & Defranceschi, 1952).

Diaminopimelic acid was found in chromatograms from all the bacteria examined with the exception of *Actinomyces* spp., *Streptomyces* spp., the Gram-positive cocci, and the one strain of *Clostridium tetani* examined. The amino acid was also found in the Myxophyceae (blue-green algae), but not in any other algae, nor in fungi, yeasts, protozoa, and plant viruses. Concentrations of diaminopimelic acid varied from about 2% (dry weight) to trace amounts of about 0·02% which were only demonstrable by grossly overloading the chromatogram with the other neutral amino-acids.

Seven unidentified spots were found. Some (such as E, F or G) occurred in chromatograms of only one type of organism, others (e.g. A, D) were distributed among certain genera. The substances responsible for spots A, E, F and G were neutral, the others moved from the centre compartment during electrodialysis but their direction of movement was not investigated. Spot F appears to correspond in position to taurine, while spot E resembles diiodotyrosine in position but not in colour (Dent, 1948).

β-Alanine (characterized by a vivid blue colour with ninhydrin and migration from the neutral compartment on electrodialysis) was observed only in certain genera of Gram-negative Eubacteriales.

α-Aminobutyric acid was found in only three organisms, all unrelated. γ-Aminobutyric acid was more widely distributed, except among the Gram-negative Eubacteriales.

The chromatograms all showed the presence of most of the amino-acids usually found in proteins; the technique used did not however separate phenylalanine from the leucine isomers, nor lysine from hydroxylysine and ornithine; histidine was seldom observed. Hydroxyproline was found once only, in a high concentration on chromatograms from the ciliate *Tetrahymena piriformis*. The general pattern of the spots showed that there were significant variations in concentrations of individual amino acids from different organisms.

Diaminopimelic acid in certain cell fractions

Table 3 represents the results of examination of hydrolysates of various types of fractions obtained from micro-organisms. No diaminopimelic acid was found in the soluble amino acid fraction from two Gram-negative bacteria and a blue green alga, although such fractions from some other bacteria are known to contain it (Work, 1949; Blass *et al.* 1951). Various soluble protein fractions from *Bact. coli* and *C. diphtheriae* contained diaminopimelic acid, as did the crude fraction remaining after separation of the insoluble cell walls. The purest preparation of *Sh. dysenteriae* endotoxin was free from diaminopimelic acid, showing that the trace found in the less pure preparation was a contaminant.

Table 3. *Distribution of diaminopimelic acid (DAP) in some cell fractions*

Organism	Fraction	Source of fraction	DAP content
Bact. coli	Ethanolic extract	Local	0
	Whole cytoplasmic contents	M. R. J. Salton	tr.
	Extractable protein	Local	tr.
Sh. shigae	Endotoxin	W. T. J. Morgan	tr.
	Endotoxin, further purified	W. T. J. Morgan	0
Anabaena cylindrica	Ethanolic extract	Local	0
Br. abortus	Ethanolic extract	Local	0
C. diphtheriae	Phenol-soluble fraction	E. S. Holdsworth	+
	Protein: ppt. 0·5 sat. Am. SO_4	Local	+
	Protein: ppt. 0·8 sat. Am. SO_4	Local	+
	Protein: ppt. 1·0 sat. Am. SO_4	Local	tr.

DISCUSSION

Identification of diaminopimelic acid

The chromatographic identification of diaminopimelic acid in acid hydrolysates of proteins is complicated by several factors. First, on chromatograms diaminopimelic acid lies near to both aspartic acid and glutamic acid; in fact, with the distortion in this region due to mineral acid ions, overlapping often occurs, especially when large amounts of hydrolysate are used in attempts to detect low concentrations of diaminopimelic acid. Cystine and ethanolamine phosphoric acid can be confused with diaminopimelic acid. Cystine is found in hydrolysates of nearly all proteins. Its behaviour on chromatograms is unpredictable; sometimes it occupies the same position as diaminopimelic acid, but usually part of the cystine is oxidized on the paper to cysteic acid (spot 18), and the remainder either gives no spot, or spots in positions 18A or 18B. Treatment with peroxide results in complete conversion of all these forms to cysteic acid, and thus avoids any danger of confusion with diaminopimelic acid. Ethanolamine phosphoric acid is a common constituent of animal tissue extracts and has also been found in yeast (Lindan & Work, 1951, Campbell & Work, 1952; Miettinen, 1951). Although its R_f value in butanol/acetic acid is slightly higher than that of diaminopimelic acid, the difference is not sufficiently great for purposes of identification. However, the acidic nature of ethanolamine phosphoric acid enables it to be separated by electrodialysis from the neutral diaminopimelic acid. The presence in micro-organisms, other than yeast, of ethanolamine phosphoric acid has not been reported, but in chromatograms of some cocci we found a spot in the expected position which was not subsequently apparent in the neutral fraction; no further examination was carried out.

In this survey the routine method employed for the identification of diaminopimelic acid consisted of two-dimensional chromatography of the peroxide-treated neutral fraction of the electrodialysed hydrolysate. Removal of all acidic ions avoided both confusion with ethanolamine phosphoric acid and interference by aspartic and glutamic acids. This enabled large amounts of material to be chromatographed, and thus revealed diaminopimelic acid in trace amounts. Two-dimensional chromatography was necessary to check completeness of electrodialysis, and to ensure identification of diaminopimelic acid. The justification for this step lies in the discovery in chromatograms of certain Actinomycetales of a spot (A) with an R_f value in collidine similar to that of diaminopimelic acid, but with a slightly higher R_f value in phenol. Had a one-dimensional chromatogram been used, this spot (A) might have been wrongly identified as diaminopimelic acid. The disadvantage of the routine use of two-dimensional chromatograms lies in their unsuitability for quantitative work on large numbers of samples; consequently we made no attempt to carry out exact estimations of diaminopimelic acid.

Provided the identity of the spot under investigation is certain, one-dimensional chromatography can be used for quantitative purposes. For the chromatographic estimation of diaminopimelic acid, Synge (see Note) used collidine as the solvent for the electrodialysed neutral amino-acid fraction, while Gendre & Lederer (1952) used butanol/formic acid after preliminary adsorption on acid alumina. We had hoped to develop the use of diaminopimelic acid decarboxylase as a quantitative method, but the enzyme proved to be inhibited by constituents of the crude hydrolysate (Dewey, 1952), and while it could be used on the electrodialysed hydrolysate, the losses involved in electrodialysis were too variable for quantitative work.

Diaminopimelic acid in bacterial cells

The function of diaminopimelic acid in the cell appears to be multiple. Present evidence suggests that it exists largely in the bound form, since it is still present in cell residues after extraction of soluble nitrogenous components (Work, 1950*a*; Blass *et al.* 1951; Pauletta & Defranceschi, 1952). Its presence in cellular proteins has been demonstrated by Gendre & Lederer (1952) and by our findings (Table 3), but it is not present in purified *Sh. shigae* endotoxin, purified diphtheria toxin or the PPD of tuberculin (Work, 1951). High concentrations of diaminopimelic acid were found by Powell & Strange (1953) in a peptide excreted by germinating spores of *B. subtilis*, and it was also present in insoluble cell wall proteins of *C. diphtheriae* and in separated cell walls of some Gram-negative organisms such as *Bact. coli* (Holdsworth, 1952; Salton, 1953). It is possible that in tough rigid cell walls, diaminopimelic acid might be playing the part of an insolubilizing cross-linking agent, in a manner similar to that played by cystine in the keratinous groups of proteins.

In some micro-organisms, diaminopimelic acid was found in the soluble amino-acid fraction which probably represents part of the cellular metabolic pool (see Work, 1949; Blass *et al.* 1951; Pauletta & Defranceschi, 1952). It is possible that the age of the culture, its treatment after harvesting or the activity of the cellular diaminopimelic acid decarboxylase (Dewey & Work, 1952) may determine whether or not diaminopimelic acid will be found among the free amino-acids. The diaminopimelic acid of the metabolic pool may not only be used for incorporation into proteins or other molecules, it may also act as a precursor of lysine; this is suggested from enzymic studies and from an examination of the nutritional requirements of various mutants of *Bact. coli* (Dewey & Work, 1952; Davis, 1952). A mechanism exists in *Bact. coli* for synthesis of large amounts of diaminopimelic acid, as is shown by accumulation of the amino-acid (250 mg./l.) in the culture fluid of a lysine-requiring mutant (Davis, 1952; Work & Denman, 1953; Wright & Cresson, 1953).

Diaminopimelic acid distribution and the classification of micro-organisms

Our survey of the distribution of diaminopimelic acid among micro-organisms, although incomplete, showed that this amino-acid was widely distributed among bacteria, but did not occur in any organisms unrelated to bacteria. No obvious relationship was found between gross diaminopimelic acid contents of bacteria and various properties which have been used in the numerous systems of bacterial classification, such as nutritional requirements, biochemical activities, Gram reaction, acid-fastness, immunological properties and pathogenicity. The absence of diaminopimelic acid from the Gram-positive cocci is one of the most striking facts which emerges from the survey; this absence contrasts with the results obtained from the other Lactobacteriaceae and the one Gram-negative coccus examined. In the Actinomycetales, there is a sharp differentiation between the mycobacteria, which contain diaminopimelic acid, and the actinomyces and streptomyces, where it is absent and another diaminodicarboxylic acid is found (Work, 1953). The presence of diaminopimelic acid in the three blue-green algae examined is of interest in view of the divergence of opinion as to the existence of a morphological similarity between the Myxophyceae and bacteria, the biochemical similarities being unquestioned (Stanier & Van Niel, 1941; Pringsheim, 1949). Our findings lend additional support to the suggestion of Stanier & Van Niel that the Myxophyceae and the Schizomycetes should be grouped into one kingdom, the Monera.

Diaminopimelic acid appears to be a cell constituent which differentiates certain bacteria not only from other bacterial species but also from all other micro-organisms. We suggest that the occurrence of diaminopimelic acid might qualify as a feature to be considered in bacterial classification. It is a physiological character directly concerned with biosynthetic aspects of cellular metabolism, and might have greater significance than many of the catabolic properties of organisms which are now used as differentiating characteristics.

Although in many cases the gross distribution of diaminopimelic acid in whole cells cannot be correlated with any obvious characteristic of the organism, it is possible that when definite fractions containing the amino-acid have been separated and studied they may be found to be directly connected with certain cellular functions. In the case of *Myco. tuberculosis*, an antigenic lipopolysaccharide fraction from human strains was found by Asselineau & Lederer (1950) to contain diaminopimelic acid only when the strain was a virulent one, although whole cells from virulent or avirulent strains contained the amino acid (Gendre & Lederer, 1952). The presence of high concentrations of diaminopimelic acid in the cell wall fractions of *Bact. coli* and *C. diphtheriae* does not imply that these fractions always represent the bulk of the amino-acid, since organisms without rigid cell walls such as myxobacteria and Myxophyceae also contain substantial amounts of it.

Amino acids other than diaminopimelic acid

The survey of two-dimensional chromatograms of the large number of micro-organisms examined enables some generalizations about the amino acid patterns to be suggested. It appears that each species has a characteristic overall amino acid composition. The recording of unidentified ninhydrin-reacting substances, and amino acids found usually only in the free state, has yielded some interesting results. For example, every strain examined in certain genera of Gram-negative Eubacteriales contained relatively high concentrations of β-alanine, but rarely showed the presence of γ-aminobutyric acid, which was found fairly frequently in other organisms. It should be emphasized that examination of crude or electrodialysed hydrolysates of whole cells would not reveal trace amounts of amino acids confined to certain cellular fractions, so that failure to record them in this survey does not imply their absence from the cell. For example, in *C. diphtheriae*, hydrolysates of whole cells did not reveal α- or γ-aminobutyric acids or β-alanine, all of which were known to occur in the soluble amino acid fraction; while hydroxyproline was found only in the insoluble fraction after removal of the soluble amino-acids (Work, 1949). Therefore, when whole cell hydrolysates are found by this technique to contain a particular amino acid which does not normally occur in proteins, the organism can be considered to contain unusually large amounts of that amino acid.

The number of unidentified spots observed seems small considering the number of organisms examined. However, the primary object of the investigation was to examine the distribution of diaminopimelic acid, not to search for other new compounds. It is noteworthy that, although some of the unidentified spots were found in chromatograms from more than one type of micro-organism, none of them had the wide distribution of diaminopimelic acid.

We are most grateful to the undermentioned for growing organisms for us (numbers are used for reference in Table 2): 1, Lt-Col. H. J. Bensted; 2, Mrs M. Bentley; 3, Dr W. A. P. Black; 4, Prof. F. Challenger; 5, Dr H. Chantrenne; 6, Dr G. C. Codner; 7, Dr R. Davis; 8, Prof. F. Egami; 9, Dr S. R. Elsden; 10, Dr G. E. Fogg; 11, Dr H. H. Green; 12, Dr P. J. Hare; 13, Dr D. W. Henderson; 14, Prof. B. C. J. G. Knight; 15, Prof. H. A. Krebs; 16, Dr R. Markham; 17, Dr G. F. Newton; 18, Dr J. R. Postgate; 19, Dr Muriel Robertson; 20, Mr A. F. B. Standfast; 21, Dr J. Tosic; 22, Dr W. E. van Heyningen; 23, Dr C. B. van Niel; 24, Dr J. M. Wiame; 25, Dr T. S. Work.

We also acknowledge cultures from: 26, Dr B. Davis; 27, Dr E. F. Gale; 28, Mr H. Proom; 29, Dr H. G. Thornton; 30, Prof. Wilson Smith.

Technical assistance was given by R. Broadman, R. F. Denman and Miss B. C. Knight.

One of us, D. L. D., was in receipt of a grant from the Rockefeller Research Fund of this Medical School.

REFERENCES

Allen, M. B. (1952). The cultivation of *Myxophyceae*. *Arch. Mikrobiol.* **17**, 34.

Asselineau, J., Choucroun, N. & Lederer, E. (1950). Sur la constitution chimique d'un lipo-polysaccharide antigenique extrait de *Mycobacterium tuberculosis* var. *hominis*. *Biochim. biophys. Acta*, **5**, 197.

Asselineau, J. & Lederer, E. (1950). Sur des différences chimiques entre des souches virulentes et non virulentes de *Mycobacterium tuberculosis*. *C.R. Acad. Sci., Paris*, **230**, 142.

Blass, J., Lecomte, O. & Macheboeuf, M. (1951). Recherches sur les aminoacids libres de *Vibrio cholerae* par microchromatographie. *Bull. Soc. Chim. biol., Paris*, **33**, 1552.

Bremner, J. M. (1950). The amino-acid composition of the protein material in soil. *Biochem. J.* **47**, 538.

Campbell, P. N. & Work, T. S. (1952). Fractionation of the nitrogenous water-soluble constituents of liver. *Biochem. J.* **50**, 449.

Campbell, P. N., Work, T. S. & Mellanby, E. (1951). The isolation of a toxic substance from agenized wheat flour. *Biochem. J.* **48**, 106.

Davis, B. D. (1952). Diaminopimelic acid and lysine. Biosynthetic interrelations of lysine, diaminopimelic acid and threonine in mutants of *Escherichia coli*. *Nature, Lond.* **169**, 534.

Dent, C. E. (1948). A study of the behaviour of some sixty amino-acids and other ninhydrin-reacting substances on phenol-"collidine" filter-paper chromatograms, with notes as to the occurrence of some of them in biological fluids. *Biochem. J.* **43**, 169.

Dewey, D. L. (1952). The metabolism of diaminopimelic acid. Thesis, London University.

Dewey, D. L. & Work, E., (1952). Diaminopimelic acid and lysine. Diaminopimelic acid decarboxylase. *Nature, Lond.* **169**, 533.

Edman, P. (1945). On the purification and chemical composition of hypertensin. *Ark. Kemi Min. Geol.* A, **22**, 1.

Fowden, L. (1951). Amino-acids of certain algae. *Nature, Lond.* **167**, 1030.

Gendre, T. & Lederer, E. (1952). Sur la présence de l'acid α,ϵ-diaminopimelic dans diverses souches de mycobactéries. *Biochim. biophys. Acta*, **8**, 49.

Holdsworth, E. S. (1952). The nature of the cell wall of *Corynebacterium diphtheriae*. Isolation of an oligosaccharide. *Biochim. biophys. Acta*, **9**, 19.

Landua, A. J., Fuerst, R. & Awapara, J. (1951). Paper chromatography of amino-acids. Effect of pH of sample. *Anal. Chem.* **23**, 162.

Lindan, O. & Work, E. (1951). The amino-acid composition of two yeasts used to produce massive dietetic liver necrosis in rats. *Biochem. J.* **48**, 337.

Miettinen, J. K. (1951). Different nitrogen fractions in normal and low-nitrogen cells of micro-organisms. *Acta chem. scand.* **5**, 962.

Pauletta, G. & Defranceschi, A. (1952). Studies on the amino-acids metabolism of *Mycobacterium tuberculosis*. *Biochim. biophys. Acta*, **9**, 271.

Powell, J. F. & Strange, R. E. (1953). Biochemical changes occurring during the germination of bacterial spores. *Biochem. J.* **54**, 205.

Pringsheim, E. G. (1949). The relationship between bacteria and myxophyceae. *Bact. Rev.* **13**, 47.

Salton, M. R. J. (1953). Studies of the bacterial cell wall. IV. The composition of the cell walls of some gram-positive and gram-negative bacteria. *Biochim. biophys. Acta*, **10**, 512.

Stanier, R. Y. & Van Niel, C. B. (1941). The main outlines of bacterial classification. *J. Bact.* **42**, 437.

Woolley, D. W., Schaffner, G. & Braun, A. C. (1952). Isolation and determination of structure of a new amino-acid contained within the toxin of *Pseudomonas tabaci*. *J. biol. Chem.* **198**, 807.

Work, E. (1949). Chromatographic investigations of amino-acids from micro-organisms. I. The amino-acids of *Corneybacterium diphtheriae*. *Biochim. biophys. Acta*, **3**, 400.

Work, E. (1950*a*). Chromatographic investigations of amino-acids from micro-organisms. II. Isolation of two unknown substances from *Corynebacterium diphtheriae*. *Biochim. biophys. Acta*, **5**, 204.

Work, E. (1950*b*). A new naturally occurring amino-acid. *Biochem. J.* **46**, v.

Work, E. (1951). The isolation of α,ϵ-diaminopimelic acid from *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*. *Biochem. J.* **49**, 17.

Work, E. (1953). The diaminodicarboxylic acids of the *Actinomycetales*. *J. gen. Microbiol.* **9**, ii.

Work, E. & Denman, R. F. (1953). The use of a bacterial culture fluid as a source of diaminopimelic acid. *Biochim. biophys. Acta*, **10**, 183.

Work, E. & Dewey, D. L. (1952). The distribution of diaminopimelic acid in micro-organisms. *Int. Congr. Biochem., Paris*, p. 98.

Wright, L. D. & Cresson, E. L. (1953). The isolation and characterization of diaminopimelic acid from the culture filtrate of an *Escherichia coli* mutant. *Proc. Soc. exp. Biol., N.Y.* **82**, 354.

Note on the Occurrence of Diaminopimelic Acid in some Intestinal Micro-organisms from Farm Animals

By R. L. M. Synge

Rowett Research Institute, Bucksburn, Aberdeenshire

Micro-organisms were analysed for diaminopimelic acid by a procedure similar to that described above by Work & Dewey. Freeze-dried preparations of the micro-organisms (which had been washed on the centrifuge with distilled water) were hydrolysed for 24 hr. in 6N-HCl at 105°. After evaporation of excess HCl *in vacuo* the residue was dissolved in water and subjected to ionophoresis in a 4-compartment diaphragm cell (Synge, 1951), maintaining the pH at 6. The contents of the specimen compartment were concentrated *in vacuo* to suitable volume; a measured amount of this solution, to which had been added 0·1% (w/v) of ammonium vanadate, was pipetted for chromatography on to the starting line of a 35 cm. tall cylinder of Munktell OB filter paper (Grycksbo Pappersbruk AB, Grycksbo, Sweden). After treatment of the spot with hydrogen peroxide (100 vol.; see Dent, 1948) the chromatogram was developed one-dimensionally (upwards) with water-commercial collidine mixture (Yorkshire Tar Distillers, Ltd., Cleckheaton). Graded known amounts (0·2–0·7 μg. N) of α,ϵ-diaminopimelic acid (isolated from *Corynebacterium diphtheriae* by Dr Elizabeth Work) were pipetted on to the paper (as hydrochloride in aqueous solution) and chromatographed in parallel without H_2O_2 treatment. After spraying with ninhydrin solution in the usual way, visual comparison was made of the unknown and control diaminopimelic acid spots, which had the lowest R_f values. A control analysis of an acid hydrolysate of 50 mg. casein gave no visible spot in the diaminopimelic acid position. On repeating with addition of 0·1 mg. diaminopimelic acid an apparent recovery of 83% was obtained. The loss was mainly into the cathode compartment of the diaphragm cell. The same recovery was assumed in calculating all the data given below. An amount of diaminopimelic acid corresponding to 0·1% or more of the N of the micro-organisms could be detected by this procedure.

The results obtained with a number of micro-organisms are given in Table 1. In agreement with Dewey & Work negative results were obtained with commercial preparations of *Torula utilis* and a *Saccharomyces* sp.

This work was undertaken after observations by Klungsøyr & Synge (see footnote, Work, 1950) of the occurrence of diaminopimelic acid in hydrolysates of rumen contents and of its absence from hydrolysates of all the usual animal feeding stuffs so far examined. It was hoped that it might serve as a means of measuring the amounts of microbial protein present in the rumen on normal diets in the same way as lysine was used as a marker with a special lysine-deficient diet containing zein by McDonald (1948). However, the differences in diaminopimelic acid content of the different micro-organisms show that changes in the flora could invalidate any estimate of total microbial protein made on this basis. Nevertheless, study of the diaminopimelic acid content of rumen contents may in future prove useful for checking claims that a particular organism has increased in numbers or become ecologically dominant. The suggestion has been made (Synge, 1952) that, in view of the high content of diaminopimelic acid in *Ruminococcus flavefaciens*, this substance may be required as a growth factor by the organism, which would explain why clostridial extracts were found by Sijpesteijn (1951) to stimulate growth while yeast extracts were less effective. The ruminococcus used in the present work was grown on a liquid medium without clostridial extract.

Table 1. *Diaminopimelic acid content of micro-organisms*

Organism	Reference	N (micro-Kjeldahl, as % of air-dry preparation)	Diaminopimelic acid (N as % total N of preparation)
Clostridium butyricum (pig-caecum strain)	Baker, Nasr, Morrice & Bruce (1951)	8·1	0·4
Cl. butyricum (sheep-rumen strain)	Masson (1950)	9·6	0·6
Cl. welchii (sheep-rumen strain)	Isolated by Miss M. J. Masson	12·5	1·6
Ruminococcus flavefaciens (strain S)	Sijpesteijn (1951)	9·3	1·0
Streptococcus faecalis (sheep-rumen strain)	Masson (1950); Moir & Masson (1952, no. 33)	8·4	None
Amylolytic streptococcus (4 sheep-rumen strains)	Macpherson (1953); cf. Macpherson & Oxford (1952)	6·6–9·3	None
Coliform xylose fermenter (culture 14)	Heald (1952*a*, *b*)	10·1	0·8

I am grateful to Dr S. R. Elsden, Dr P. J. Heald, Miss Margaret Macpherson and Miss Marjorie Masson who provided cultures for this work, and to Mr J. Wood for technical assistance.

REFERENCES

BAKER, F., NASR, H., MORRICE, F. & BRUCE, J. (1951). Bacterial breakdown of structural starches and starch products in the digestive tract of ruminant and non-ruminant animals. *J. Path. Bact.* **62**, 617.

DENT, C. E. (1948). A study of the behaviour of some sixty amino-acids and other ninhydrin-reacting substances on phenol-'collidine' filter-paper chromatograms, with notes as to the occurrence of some of them in biological fluids. *Biochem. J.* **43**, 169.

HEALD, P. J. (1952*a*). The fermentation of pentoses and uronic acids by bacteria from the rumen contents of sheep. *Biochem. J.* **50**, 503.

HEALD, P. J. (1952*b*). The metabolism of glucuronic acid by xylose-fermenting coliform bacteria. *Biochem. J.* **52**, 378.

McDONALD, I. W. (1948). The extent of conversion of food protein to microbial protein in the rumen of the sheep. *J. Physiol.*, **107**, 21*P*.

MACPHERSON, M. J. (1953). The isolation and identification of amylolytic streptococci from the rumen of the sheep. *J. Path. Bact.* **66**, 95.

Journal of General Microbiology, Vol. 9, No. 3

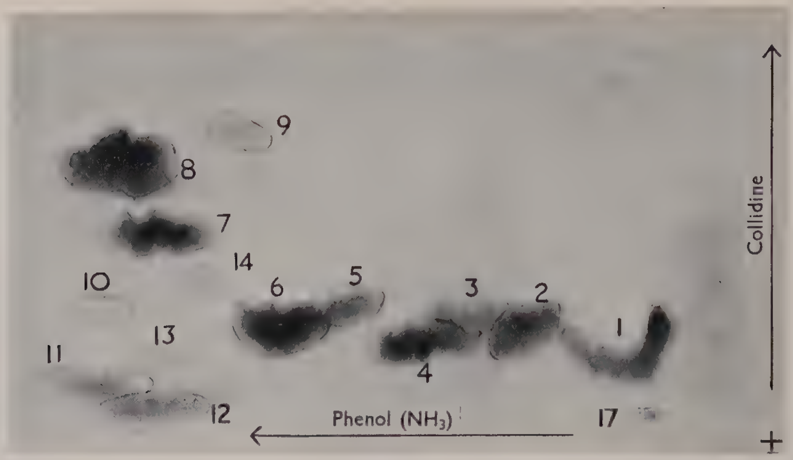

9
8
7
14
10
6
5
3
2
13
1
11
4
12
Phenol (NH₃)
17
Collidine
+

Fig. 1

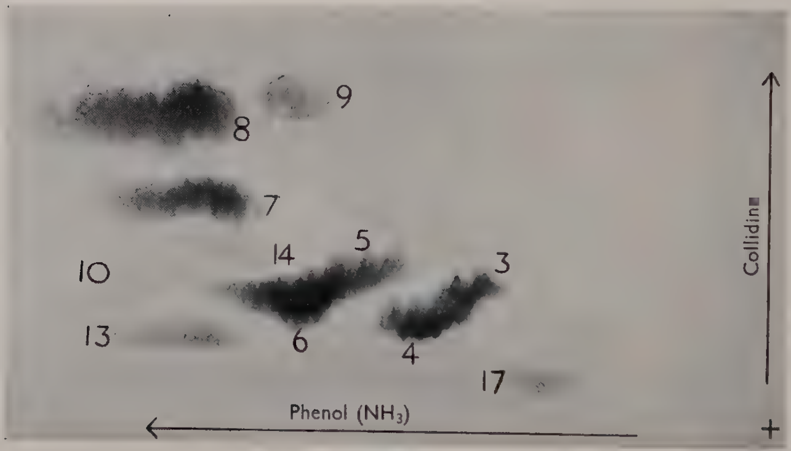

9
8
7
5
14
3
10
13
6
4
17
Phenol (NH₃)
Collidine
+

Fig. 2

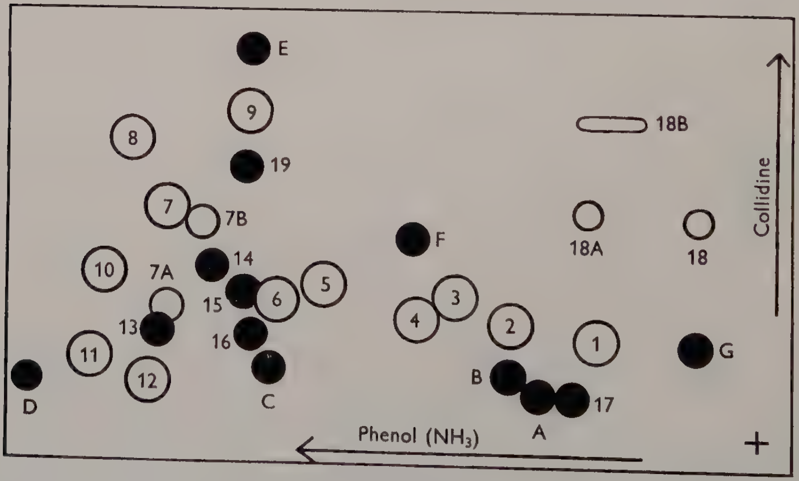

E
9
18B
8
19
Collidine
7
7B
F
18A
18
10
7A
14
5
15
6
3
4
2
13
16
1
G
11
12
B
D
C
17
A
Phenol (NH₃)
+

Fig. 3

R. L. M. Synge—Diaminopimelic acid in micro-organisms. Plate 1

Macpherson, M. J. & Oxford, A. E. (1952). The use of the Neufeld capsular swelling reaction in the identification of rumen streptococci *in situ*. *J. gen. Microbiol.* **7**, ii.

Masson, M. (1950). Microscopic studies of the alimentary micro-organisms of the sheep. *Brit. J. Nutr.* **4**, viii.

Moir, R. J. & Masson, M. J. (1952). An illustrated scheme for the microscopic identification of the rumen micro-organisms of sheep. *J. Path. Bact.* **64**, 343.

Sijpesteijn, A. K. (1951). On *Ruminococcus flavefaciens*, a cellulose-decomposing bacterium from the rumen of sheep and cattle. *J. gen. Microbiol.* **5**, 869.

Synge, R. L. M. (1951). Non-protein nitrogenous constituents of rye grass: ionophoretic fractionation and isolation of a 'bound amino-acid' fraction. *Biochem. J.* **49**, 642.

Synge, R. L. M. (1952). A discussion on symbiosis involving micro-organisms. *Proc. roy. Soc.* B, **139**, 205.

Work, E. (1950). Chromatographic investigations of amino-acids from micro-organisms. II. Isolation of two unknown substances from *Corynebacterium diphtheriae*. *Biochim. biophys. Acta*, **5**, 204.

EXPLANATION OF PLATE

Fig. 1. Chromatogram of crude hydrolysate of *Rhodopseudomonas spheroides* (1·6 mg. dry wt.). For key to numbers see Fig. 3.

Fig. 2. Chromatogram of H_2O_2 treated electrodialysed hydrolysate of *Rhodopseudomonas spheroides* (3·2 mg. dry wt.). For key to numbers see Fig. 3.

Fig. 3. Key to ninhydrin-reacting spots found on chromatograms of hydrolysates of micro-organisms. First solvent phenol (NH_3 atmosphere), second solvent, collidine/lutidine. *Clear circles* represent amino-acids which are normal protein constituents. 1, aspartic acid; 2, glutamic acid; 3, serine; 4, glycine; 5, threonine; 6, alanine; 7, valine; 7A, methionine sulphoxide; 7B, methionine sulphone; 8, leucines, methionine and phenylalanine; 9, tyrosin; 10, proline; 11, arginine; 12, lysine; 18, cysteic acid; 18A and 18B, some positions occupied by cystine or its oxidation products (spot 17, below, also corresponds to cystine); 19, glucosamine.* *Black circles* represent additional spots not normally found in bacterial proteins. 13, γ-aminobutyric acid; 14, α-aminobutyric acid; 15, hydroxyproline; 16, β-alanine; 17, ethanolamine phosphoric acid and diaminopimelic acid.* A,* B, C, D, E, F, G, unidentified; A, E, F and G are neutral.

* R_f in phenol variable, according to pH.

(*Received* 8 *May* 1953)

STOCKER, B.A.D., ZINDER, N.D. & LEDERBERG, J. (1953). *J. gen. Microbiol.* 9, 410–433.

Transduction of Flagellar Characters in Salmonella

BY B. A. D. STOCKER*

Department of Bacteriology and Immunology, London School of Hygiene and Tropical Medicine, Keppel Street (Gower Street), London, W.C. 1

AND N. D. ZINDER† AND J. LEDERBERG

Department of Genetics, University of Wisconsin, Madison, Wisconsin, U.S.A.

SUMMARY: Phage lysates of a salmonella strain can transduce flagellar characters from that strain to a second strain which absorbs the phage. Non-motile strains treated with lysates of other strains produced stable motile forms detected as spreading swarms in semi-solid agar.

O strains of known species produced swarms with the H antigens characteristic of their own species, and the species of previously untypable O isolates could be inferred from the H antigens of their induced swarms.

The production of swarms by O strains when treated with lysates of other O strains divided nine *Salmonella typhimurium* O strains into six interacting groups; there are therefore at least six non-homologous genetic factors, mutation of any of which may cause absence of flagella, thus masking other genes which control H antigenic specificity.

Two flagellated but non-motile strains produced motile forms when treated with lysates of other strains, or of each other. At least two genes concerned with locomotor function are thus recognized. All the motile derivatives of one O strain spread very slowly through semi-solid agar; the ability to spread rapidly could be transduced to them by a second exposure to a lysate.

In exceptional instances two flagellar characters were simultaneously transduced: three O strains sometimes acquired a flagellar antigen from the donor strain at the same time as the H character; another O strain sometimes acquired simultaneously flagellation and the slow-spreading character.

There are therefore many distinct genes controlling the presence, antigenic character and function of the flagella. They may form part of an ordered gene array in which pairs of genes which are sometimes simultaneously transduced by a single phage particle are presumably closely linked.

Hereditary properties can be transferred or *transduced* from one salmonella strain to another by the action of cell-free culture filtrates (Zinder & Lederberg, 1952; Lederberg, Lederberg, Zinder & Lively, 1951). Filtrates of a salmonella strain lysed by a suitable phage were found to contain an agent which transferred various characters from the lysed (donor) strain to a small proportion of the cells of a recipient strain exposed to the filtrate. As a rule a maximum of only one per million recipient cells acquired a new character, and the simultaneous transfer of two characters from the donor strain to a single cell could not be demonstrated. Cells which had thus acquired a new character by transduction transmitted it to all their progeny through an unlimited number of generations, so far as was tested. The phenomenon is believed to result from the transference of genetic factors from the donor strain to some cells of the recipient strain. Hereditary changes or *transformations* of strains of pneumococci and of *Haemophilus influenzae* may be effected by the action of cell-free lysates of other strains (Griffith, 1928; Alexander & Leidy, 1951); these changes also are now considered to result from the transfer of genetic material from the lysed strain to the recipient strain. In these organisms the transforming activity of lysates depends on their content of undepolymerized deoxyribonucleic acid, and is destroyed by the action of deoxyribonuclease (Avery, MacLeod & McCarty, 1944). The transducing activity of salmonella lysates, however, is unaffected by this enzyme, and appears to be associated with the phage particles present in the lysate. It is believed that in salmonella transduction some of the genetic material of a donor cell is incorporated into some of the phage particles which cause its lysis, and that such genetic material is carried by a phage particle into a recipient cell, where it replaces homologous genetic material. The phages concerned neither lyse nor kill a high proportion of cells which absorb them, even in the case of sensitive strains; that is, they are of the kind called temperate (Lwoff, 1953) or symbiotic (Boyd, 1950).

---

* Present address: Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W.1.

† Present address: Rockefeller Institute for Medical Research, New York 21, N.Y., U.S.A.

Transduction differs from two other kinds of alteration of the hereditary properties of bacterial strains which may result from exposure to phage, namely the selection of phage-resistant mutants, and the establishment of lysogenicity. In each of these processes strains having new characters, i.e. phage resistance or carriage, and sometimes other correlated characters, are obtained; but the kind of altered strain obtainable is determined only by the phage used, and does not depend on the character of the host on which this phage was grown, as in transduction. The relation of transduction in salmonellas to genetic recombination in *Bacterium coli* has been discussed by Zinder & Lederberg (1952) and Lederberg *et al.* (1951). It must suffice here to recall that recombinants cannot be obtained under conditions which prevent the possibility of cell to cell contact of the two parent strains, that is, filtrates are inactive, and that when the parent strains differ in several characters many of the recombinants obtained derive several characters from *each* parent strain.

The experiments described in the present paper were designed on the assumption that the transducing activity of filtrates or pasteurized supernatants of salmonella cultures resides in the phage particles which they contain; and they have been interpreted on the premise that the range of characters which a filtrate can transduce is determined by the genetic constitution of the cells in which these phage particles were formed.

The flagella of Salmonellas have been intensively studied, largely because of their importance in the identification of strains pathogenic for man or animals. Some of the available information may be briefly summarized here. All but two species, or serotypes as some authorities prefer, of salmonella normally have flagella, by means of which they are motile. In some environments flagella are not formed, e.g. on media containing phenol (Braun, 1918). Furthermore, strains may undergo spontaneous mutations affecting the presence or abundance of flagella. Non-flagellated variants (known as O forms, in distinction from flagellated or H forms) have frequently arisen in laboratory cultures, and similar strains have been isolated from natural sources. Such O forms retain the non-flagellated character on subculture, but occasionally produce H mutants which themselves breed true. H mutants may be detected and isolated by inoculating the O culture into semi-solid agar, since any motile cells present can spread to parts of this medium inaccessible to non-motile cells, which remain at the site of inoculation. Stable O forms have also been obtained from H cultures by exposing them to the action of a phage which lyses only flagellated cells (Sertic & Boulgakov, 1936).

The flagella of salmonellas are efficient antigens, and the species described in the genus are to a large extent differentiated by the antigenic specificity of their flagella. The major antigenic relationships are recorded in the Kauffmann-White schema (Salmonella Sub-Committee, 1949; Kauffmann, 1951). In this schema recognizable flagellar (H) antigens or antigenic 'components' are denoted by the small arabic numerals 1 to 11, and by small letters from a to z and from z_1 to z_{36}, the z in the z-subscript series not denoting any antigenic relationship but merely the exhaustion of the alphabet. Some species have flagella indicated in the schema by several symbols, for instance g, p, u in the case of *Sal. rostock*. In the present work it has been assumed that such a group of symbols represents the antigenic reactivity of only a single kind of antigen molecule, present in or constituting the flagella of the organism concerned.

Andrewes (1922, 1925) showed that in many species of salmonella the flagella may show either of two alternative antigens, and that cells in one 'phase' produce progeny predominantly in that phase but also a proportion of descendents in the opposite phase, which in turn breed true for the most part but produce a proportion of cells in the original phase. The rates of spontaneous change of phase have been measured in *Sal. typhimurium* and found to be very high, ranging between 9×10^{-4} and 10^{-5} per bacterium per generation time (Stocker, 1949). Most of the flagellar antigens of the genus fall into two groups, such that the alternative antigens of diphasic organisms include one from each group.

The large number of known antigenic characters, the possibility of selecting either for motility or for the absence of flagella and the extent to which flagellar characters have been investigated make them favourable material for genetic studies. Zinder & Lederberg (1952) showed that flagellar characters could be transduced, for they were able to replace the characteristic flagellar antigen d of a strain of *Sal. typhi* by antigen i from a strain of *Sal. typhimurium*. The present paper describes further investigations of the transduction of various flagellar characters.

METHODS AND MATERIALS

Phage PLT 22, described by Zinder & Lederberg (1952), was used for making transducing lysates, and will be referred to in the present paper as phage 22. Brig. J. S. K. Boyd informs us that this phage falls into group A1 of his classification of the symbiotic phages of *Sal. typhimurium* (Boyd, 1950). The host strain used for routine propagation was *Sal. typhimurium* strain TM 2 (strain LT 2 of Zinder & Lederberg, 1952).

To obtain a transducing preparation of a salmonella strain, phage 22 was propagated on it in broth, under conditions such as to avoid the inhibition of lysis which occurs when the ratio of added phage to bacteria is high (Boyd, 1951). Broth cultures in which there was at least partial clearing were centrifuged, and the supernate was heated at 60° for 30 min., to kill all bacteria without affecting the phage. When phage was being grown on a new strain, steps were taken to ensure that the carry-over of inoculum-phage, grown on some other host, was negligibly small. Satisfactory lysates generally gave plaque counts of about 3×10^9/ml. All phage preparations were tested for sterility, both at the time of preparation and when used.

Table 1. *Strains used, characters, and whence obtained*

Our strain number	Description	Whence obtained and donor's strain number	References
TM2	*Sal. typhimurium* H	Lilleengen, No. 85 = LT2 of Zinder & Lederberg	Lilleengen (1948), Zinder & Lederberg (1952)
SW533	*Sal. paratyphi-B* H (phase 2 variant of a *java* strain)	Edwards, No. 157	Edwards & Bruner (1946)
SW534	*Sal. paratyphi-B* O	Obtained from SW533 by action of phage VIII–113	Sertic & Boulgakov (1936)
SW541	Salmonella O of Group B	Kauffmann, No. 223 = Cop. O 1810	Kauffmann (1947)
SW543	*Sal. paratyphi-B* O	Kauffmann, No. 248 = OB 19	Kauffmann (1947)
SW544	*Sal. typhimurium* O	Schütze, Glasgow O, *via* NCTC, 3047; = Strain OB of Felix	Schütze (1930), Felix (1930)
SW545	Salmonella O of Group B	Edwards, 4937/50	.
SW548	Salmonella O of Group B	Edwards, 2294/49	.
SW549	Salmonella O of Group B	Edwards, 117/51	.
SW553	Salmonella O of Group D	Edwards, 1520/51	.
SW573	*Sal. typhimurium* O	Leifson, R20 1/2	Friewer & Leifson (1952)
SW578	*Sal. typhimurium* non-motile H strain	Leifson, FM 61/63	Friewer & Leifson (1952)
SW580	*Sal. typhimurium* non-motile H strain	Leifson, FM 59/64	Friewer & Leifson (1952)
SL13	*Sal. paratyphi-A*, O variant of an H strain	Felix, A17689 (O form)	Felix (1952*a*)
SL15	Salmonella O of Group B	Taylor, Sal. 1067/52	.
SL18	Salmonella O of Group B	Taylor, Sal. 1565/52	.
SL20	Salmonella O of Group B	Taylor, Sal. 1878/52	.
SL28	Salmonella O of Group B	Taylor, Sal. 1775/52	.
SL47	Salmonella O of Group B	Taylor, Sal. 2280/52	.
SL48	Salmonella O of Group B	Taylor, Sal. 2035/52	.
SL51	Salmonella O of Group B	Taylor, Sal. 2364/52	.
SL54	Salmonella O of Group B	Taylor, Sal. 2460/52	.
SL55	Salmonella O of Group B	Taylor, Sal. 2462/52	.
SL78	*Sal. typhi*, O variant of an H strain	Felix, O901	Felix (1930)

The strains used are listed in Table 1. Most of the non-flagellated (O) strains were received from various workers. Other O strains used were non-flagellated mutants which we obtained from H strains by exposure to phage VIII–113 of Sertic & Boulgakov (1936); this phage attacks many salmonella strains, but only when they have flagella.

Flagellar and somatic antigens were tested for by slide agglutination, using suitably diluted absorbed sera. All strains of critical importance were submitted either to Dr P. R. Edwards, at the Communicable Disease Center, U.S. Public Health Service, Chamblee, Georgia, or to Dr Joan Taylor, at the Salmonella Reference Laboratory of the Public Health Laboratory Service, Colindale, London, for detailed serological analysis.

To detect the presence of any motile cells in non-motile cultures, arising either by spontaneous mutation or as a result of transduction, loopfuls of broth culture, or the whole of the deposit from a centrifuged culture, were placed on a circumscribed area of the surface of a medium containing 0·4 % agar (Difco) and 8 % gelatin (Difco) in a nutrient broth base (Edwards & Bruner, 1942). This medium is semi-solid at 37°, so that motile strains spread through it as they grow; but because of its high gelatin content it solidifies at room temperature. To economize materials, this medium was used in 5 cm. Petri dishes, about 10 ml. per dish. Inoculated plates were incubated uninverted for 10–18 hr. at 37°, allowed to solidify at room temperature and examined for swarms or other evidence of bacterial movement by inspection with a plate microscope and oblique transmitted light. In plates incubated for 10 hr. swarms were usually only about 3 cm. in diameter and, if not numerous, were still discrete. But after 18 hr. incubation even a single swarm would generally have spread right across the plate.

RESULTS

Behaviour of untreated non-motile strains grown on semi-solid medium

The growth of most of the non-motile strains on semi-solid agar was strictly confined to the site of inoculation, even when incubation at 37° was continued for 48 hr. or longer (Pl. 1, fig. 1). Some strains, however, gave rise to spontaneous swarms which on subculture were found to consist of stable, motile, H-agglutinable mutants. In many such strains this mutation was a rare event, so that only a few plates showed swarms. In others the mutation was more frequent, so that most or all plates showed spontaneous swarms, but they usually appeared rather late, that is later than the 12th hour of incubation; this presumably indicates that only after several hours of incubation was the population of cells large enough, in relation to the mutation rate, for there to be more than a small probability of a mutation occurring. A few O strains were discarded because of their inconveniently high rate of mutation.

Sal. typhimurium O strain SW 545 grew on the surface of the semi-solid medium at the site of inoculation and also produced large numbers of microcolonies, singly or in small groups, 1 mm. or less below the surface of the agar (Pl. 1, fig. 2). Subculture from these colonies yielded only stable O forms similar to the original strain, and indeed H mutants have never been obtained from strain SW 545. This phenomenon is being further investigated, but it seems probable that it results from the spontaneous occurrence in this strain of a very small fraction of cells which are motile, and therefore migrate through the agar, but which produce only non-motile progeny. Micro-colonies separate from and deep to the site of inoculation have been seen in several other O strains, but in smaller numbers than in SW 545.

The centre, and therefore presumed point of origin, of a swarm was frequently occupied by an area a few millimetres in diameter in which there was a dense crowd of micro-colonies, of diameters inversely related to their distance from the centre of the area. This appearance, which is illustrated in Pl. 1, fig. 3, will be termed a 'flare'. Numerous single-colony isolations from flares crowded with micro-colonies always gave stable *motile* cultures similar to those obtained from the periphery of the swarm, which indicates that the micro-colonies consist of cells which give rise to motile progeny. Flares have also been obtained by inoculating a few cells of certain partly rough motile strains on semi-solid agar. The mechanism of the formation of micro-colonies near the point of origin of swarms is not clear; the trapping of motile cells amongst agar fibrils perhaps plays a part, but partial roughness or other physiological or genetic factors which hinder the progression of the initial motile cells may be involved.

The appearance of non-motile strains grown on semi-solid agar has remained constant from experiment to experiment. When a strain was treated with phage 22 propagated on that same strain, the behaviour of the phage-treated cells, as to production of swarms or isolated deep colonies, was exactly the same as that of the untreated culture. This would be predicted if phage in itself has no effect on hereditary properties, such as motility, but can carry across genetic material from its last host; when the genetic material of the last host and the recipient is identical, its transfer from the one to the other will have no detectable effect.

Behaviour of non-motile strains treated with transducing lysates and incubated on semi-solid agar

In tests for transduction of motility a broth culture of a non-motile strain was incubated with an equal volume of a lysate for 30 min. to allow phage adsorption. Three loopfuls of the mixture, or the whole of the deposit obtained by centrifuging 1 ml. of the mixture, were then placed on semi-solid agar and incubated at 37° for 10–18 hr. The appearances produced when transduction of motility occurred are illustrated in Pl. 1, fig. 4, which may be compared with Pl. 1, fig. 1, which shows the same recipient strain plated without phage treatment. With combinations such as that illustrated, the inoculated area was occupied by dense growth, and surrounded by a wide border of confluent swarming which extended throughout the depth of the agar; when a smaller number of phage-treated cells were inoculated, this continuous border was replaced by discrete swarms (Pl. 1, fig. 5). The centres of the swarms were sometimes marked by flares, as described in the previous section. Unlike spontaneous swarms, these induced swarms were for the most part well developed by the 12th hour of incubation. Subculture from the edge of a swarm always gave a pure growth of motile organisms, and motility was retained on further subculture. The growth obtained by subculture from the swarms differed from the parent non-motile strain by its motility and H agglutinability, and, so far as tested, in no other way, except that it was commonly but not invariably lysogenic for, and resistant to, phage 22. The occurrence of some colonies of non-lysogenic sensitive cells, which however were motile, indicates that some of the offspring of cells which acquire exogenous hereditary material do not contain descendants of the phage particle which imported the genetic material.

The trail phenomenon in transduction of motility

Many combinations of phage and recipient non-motile cells produced not only swarms but also groups of micro-colonies deep in the agar and at a distance from the site of inoculation (Pl. 1, fig. 6). These groups were generally more numerous than the swarms, and if the treated cells were appropriately diluted before inoculation, groups of micro-colonies alone were obtained (Pl. 2, fig. 7). The groups of micro-colonies had an elongated form, the unbranched line about which the colonies were scattered extending from a point in the inoculated area downwards into the agar and laterally for a distance of less than 1mm. to 20 mm. These linear groups of micro-colonies will be referred to as 'trails'. The number of colonies in a trail varied from 4 to 50, or even more in plates incubated for 48 hr. Subculture from these colonies has yielded only *non-motile* forms, similar to the parent strain; yet the distance of the micro-colonies from the site of inoculation, and their position in the depths of the medium, make it clear that the cell from which each colony grew must have reached the site of the colony by active movement. We suggest that trails result from abortive transduction, arising as follows: if a gene carried into an O cell is dominant to that responsible for the absence of flagella, the cell will become motile; but if the exogenous gene fails to replace the homologous gene in the (hypothetical) chromosome, it will not be replicated, so that at cell division there will be produced one cell containing the foreign gene and therefore motile, and one cell lacking it, and therefore non-motile. Thus at any time there will be only one motile cell containing the foreign gene, and its path through the agar will be marked by a trail of micro-colonies growing from the non-motile daughter cells produced each time it divides. Experiments to test this hypothesis are in progress. In this paper only the occurrence of swarms made up of stable motile forms has been recorded as evidence of transduction of motility; however, every combination of lysate and non-motile recipient strain which, when first tested, produced trails only, has produced swarms when the test has been repeated with larger numbers of treated cells.

Transduction of flagellation to O strains of known species

In the genus *Salmonella*, species are distinguished chiefly or entirely by their somatic and flagellar antigens, both flagellar antigenic phases of diphasic organisms being accorded taxonomic weight; some authorities consider that these antigenically differentiated forms should be called serotypes rather than species, but in the present paper we have for convenience retained the use of binomials and the designation 'species'. Most of the non-flagellated strains used by us had somatic antigens IV and XII, and thus fell into group B of the Kauffmann-White schema. In this group many species are differentiated solely by their H antigens, and it might seem that strains lacking flagella could not be assigned to species. However, some of our O strains were *in vitro* variants of H strains of known flagellar antigenic constitution, and could be regarded as belonging to the species indicated by the H antigens of their parent strains. Some of these O strains of known species produced spontaneous H mutants, which in every case resembled the original H parent both as to the presence or absence of a second phase, and in flagellar antigen or antigens. For instance, we were able to isolate motile mutants from *Sal. typhi* strain O 901; these H mutants had flagellar antigen *d* and were monophasic, like the classical *Sal. typhi* strain H 901 from which Felix and Olitzki isolated the O variant strain in 1925 (Felix, 1930). Strain O 901 has been extensively employed as an O agglutinogen and for the detection of O antibodies, and Felix & Pitt (1951) stated that no reversion to the H form had then been recorded; our recovery of spontaneous motile variants from it indicates that incubation on semi-solid agar is an extremely sensitive method for detecting motile cells arising in non-motile cultures. Thus at least some O mutant strains retain the potentiality of producing flagella of the antigenic type characteristic of their parent H forms; on the gene theory, this indicates the existence of one or more genes determining flagellar antigenic specificity which persist unaltered when the strain ceases to produce flagella, owing to mutation of some other gene.

Some O strains had been isolated from natural sources as O forms; some of these produced spontaneous H mutants, and in view of the findings described above were assigned to the species indicated by the antigens of their H mutants. In two cases an O strain had been originally detected as one component in a culture which also contained an H strain apparently identical with its O companion except for the presence of flagella; it seemed probable that in each case the O and H forms had arisen from a common ancestor, and these O strains were therefore assigned to the same species as their H companions.

A number of O strains having somatic antigen XII, and of known or suspected latent flagellar antigenic formula were thus available. All were treated with phage 22 grown on *Sal. typhimurium* TM 2, and incubated on semi-solid agar; control plates were inoculated with untreated cells. With the exception of *Sal. typhimurium* O strain SW 573, whose behaviour is discussed later, all the treated strains produced swarms. Some strains also produced spontaneous swarms; but in these strains treatment with the lysate caused an obvious increase in the number of swarms, and the induced swarms appeared earlier than the spontaneous ones. The growth from induced swarms was plated out, and the flagellar antigens of discrete colonies were tested by slide agglutination; alternative phases if not otherwise detected were sought by inoculation into semi-solid agar containing homologous H antibody. The results of these tests are shown in Table 2, which also records the H antigens of the related H strains (parent, mutant or twin component of original mixed culture). It will be seen that every O strain gave induced swarms with the same H antigenic constitution as its related H strain(s). Many of the O strains used were considered to be *Sal. typhimurium*, that is the same species as the donor strain (TM2). But when the recipient O strain was of some other species, induced motile forms were obtained which had the H antigens believed to be latent in the recipient strain.

Table 2. *Salmonella O strains of known species: H antigen(s) of parent H strains, of spontaneous H mutants, and of H forms induced by treatment with a lysate of* Sal. typhimurium *strain TM2*

			Spontaneous (mutative) swarms		Swarms induced by lysate of strain TM2	
O strain	Species	H antigen(s) of parent H strain	Rate	H antigens	Number	H antigens
SW544	*Sal. typhimurium*	—	Rare	*i*; 1, 2, 3	++	*i*; 1, 2, 3
SW548	*Sal. typhimurium*	—	Rare	*i*; 1, 2, 3	++	*i*; 1, 2, 3
SW549	*Sal. typhimurium*	—	Common	*i*; 1, 2, 3	++	*i*; 1, 2, 3
SW573	*Sal. typhimurium*	*i*; 1, 2, 3	Nil	.	0	.
SL55	*Sal. typhimurium*	—	Rare	*i*; 1, 2, 3	++	*i*; 1, 2, 3
SL13	*Sal. paratyphi-A*	*a*	Nil	.	+	*a* (most) *i* (some)
SW543	*Sal. paratyphi-B* (fixed phase 1)	*b*	Rare	*b*	++	*b* (most) *i* (some)
SW534	*Sal. paratyphi-B* (fixed phase 2)	1, 2	Common	1, 2	+	1, 2
SL78	*Sal. typhi*	*d*	Common	*d*	++	*d*

H antigens of parent strain ; — indicates strain isolated from natural source as O form. In the case of strains SW543 and SW573, the H antigens recorded are those of the H component of a mixed culture, i.e. those of the presumed parent strain. Common = spontaneous swarms on most plates incubated for 18 hr. Rare = spontaneous swarms on a few plates incubated for 18 hr. Nil = no spontaneous swarms seen in repeated tests.

Thus these O strains when made motile by transduction of flagellation produced H forms with the H antigenic constitution known or suspected to be latent in the O strain, even when it could not be demonstrated by the spontaneous production of H mutants. The evidence from transduction, therefore, supports the hypothesis that O strains retain the H-antigen-determining genes of their ancestors, and lack flagella because of some mutation not affecting these genes; the H antigen genes manifest themselves when the flagella reappear, either as a result of a spontaneous mutation of some other gene, or by transduction of some other gene.

The experimental technique used showed that all these O strains when treated with a lysate produced swarms having the H antigenic structure latent in the recipient strain, but did not exclude the possibility that they might in addition produce swarms having H antigens characteristic of the donor strain on which the transducing phage had been propagated; this could only be detected when donor and recipient strains had different H antigens. Some O strains did in fact produce a minority of swarms with H antigens characteristic not of the recipient O strain but of the donor strain. This phenomenon is described in detail in a later section; but as in these strains swarms having exogenous H antigens were always accompanied by an excess of swarms having intrinsic H antigens, the argument for the presence of H antigen determinants in O strains is not invalidated.

Transduction of flagellation to O strains of unknown species

A number of smooth O strains were available which had been isolated from nature in pure culture in the O form, and which did not produce spontaneous H mutants even in repeated tests. Some were *Sal. gallinarum* or *Sal. pullorum*, species in which only the O form is known; these strains were discarded. The others could not be assigned to species. All these strains were treated with a phage 22 lysate of *Sal. typhimurium* strain TM2; certain strains assigned to group C of the Kauffmann-White schema were unaffected, presumably because they lacked somatic antigen XII, which is believed to be required for the adsorption of phage 22. All the other O strains tested produced swarms. The motile forms were isolated and their H antigens determined (Table 3). (In some instances more than one O strain had been isolated from the same focus of food-poisoning, etc. In such cases all the strains were tested, and related O strains always produced H forms with the same H antigens; but only a single representative of each set has been included in Table 3.) Most of the O strains produced swarms having H antigens $i \leftrightarrow 1, 2, 3$, that is, those found in *Sal. typhimurium*, the species of the donor strain; others gave swarms having other H antigens, which, in conjunction with their O antigens, characterized them as *Sal. paratyphi-B*, *Sal. dublin* or *Sal. heidelberg*. When the derived strain had neither antigen *i* nor 1,..., it seemed reasonable to conclude that its H antigen(s) must have been latent in the recipient O strain. When the induced H form showed antigen(s) also present in the donor strain the evidence was equivocal, since the recipient strain might perhaps have received from the donor strain both an H antigen determinant and a gene for flagella production. The O strains concerned were therefore retested using lysates of various strains having neither antigen *i* nor antigen 1,.... In all cases the swarms thus obtained had the same H antigens as before (Table 3). The O strains concerned therefore have latent H antigenic constitutions which, with their O antigens, indicate their species as *Sal. typhimurium*, *Sal. paratyphi-B* or *Sal. heidelberg* respectively.

Although the Salmonella species concerned are defined by their O and H antigens, certain other properties show a high degree of correlation. For instance, a phage-typing scheme for *Sal. paratyphi-B* has been devised (Felix & Callow, 1943, 1951) and a provisional scheme for *Sal. typhimurium* is also in use, though it has not yet been published in detail (Felix, 1951). All our O strains of group B were submitted to Dr A. Felix at the Central Enteric Reference Laboratory and Bureau, Colindale, London, and the phage types reported by him are recorded in Table 3. All strains indicated as *Sal. paratyphi-B* or *Sal. typhimurium* by their reactions to the typing phages were assigned to the corresponding species by the results of transduction. Two strains whose reactions to the phages neither indicated nor excluded diagnosis as *Sal. paratyphi-B* or *Sal. typhimurium* were diagnosed *Sal. typhimurium* and *Sal. heidelberg*, respectively, by the results of transduction. Strain SL51, diagnosed *Sal. paratyphi-B* by both methods, was the only strain which produced the 'slime-wall' characteristic of that species. We conclude that the unmasking of latent H antigens by transduction of flagellation provides a valid method for determining the species of stable O strains of *Salmonella.*

Table 3. *Salmonella O strains of unknown species: H antigens of swarms induced by lysates of* Sal. typhimurium *and of Salmonella lacking H antigens* i *and* 1...; *reaction to* Sal. paratyphi-B *and* Sal. typhimurium *typing phages of Felix & Callow*

		H antigens of swarms induced by lysates of			
Strain number	O antigens	*Sal. typhimurium* strain TM2	Salmonella lacking H antigens *i* and 1...	Species indicated by O and induced H antigens	Reaction of O strain to typing phages
SW541	IV, XII	*i*; 1, 2, 3	*i*; 1, 2, 3	*Sal. typhimurium*	Not identifiable
SW545	IV, V, XII	*i*; 1, 2, 3	*i*; 1, 2, 3	*Sal. typhimurium*	*Sal. typhimurium* Type 2
SL15	IV, XII	*i*; 1, 2, 3	*i*; 1, 2, 3	*Sal. typhimurium*	*Sal. typhimurium* Type 2*a*
SL18	IV, V, XII	*i*; 1, 2, 3	*i*; 1, 2, 3	*Sal. typhimurium*	*Sal. typhimurium* Type 2*a*
SL20	IV, V, XII	*i*; 1, 2, 3	*i*; 1, 2, 3	*Sal. typhimurium*	*Sal. typhimurium* Type 2*a*
SL47	IV, XII	*i*; 1, 2, 3	*i*; 1, 2, 3	*Sal. typhimurium*	*Sal. typhimurium* Type 2*a*
SL48	IV, V, XII	*i*; 1, 2, 3	*i*; 1, 2, 3	*Sal. typhimurium*	*Sal. typhimurium* Type 2*a*
SL54	IV, V, XII	*i*; 1, 2, 3	*i*; 1, 2, 3	*Sal. typhimurium*	*Sal. typhimurium* Type 4
SL51	IV, V, XII	*b*; 1, 2	*b*; 1, 2	*Sal. paratyphi-B*	*Sal. paratyphi-B* Type 2
SL28	IV, V, XII	*r*; 1, 2, 3	*r*; 1, 2, 3	*Sal. heidelberg*	Not identifiable
SW553	I, IX, XII	*g,p* (most) *i* (some)	—	*Sal. dublin*	—

The salmonellas lacking antigens *i* and 1... were strains of *Sal. abortus-equi* (H antigen *e, n, x*) and *Sal. abony* (H antigens *b*; *e,n,x*). — indicates test not made. The presence or absence of O antigen I in O strains of group B has not been recorded because of the presence of colonies with and without this antigen within a single strain.

Action of lysates of O strains on other O strains

If all salmonella O strains (of species which are normally flagellated) lacked flagella from a common cause, that is mutation of the same gene, a lysate of one O strain would not be expected to confer motility on any other O strain, for the same reason that an O strain is not made motile by phage grown on itself. But if absence of flagella is due to different causes, that is mutations of non-homologous genes, in different O strains, then an O strain of one kind might produce H forms when treated with a lysate of an O strain of a different kind, for the ineffective determinant in the recipient strain could be replaced by its normal homologue from the donor strain. A situation of this sort was found by Zinder & Lederberg (1952); four galactose-non-fermenting mutants of a strain of *Sal. typhimurium* fell into two classes, a mutant of one class producing galactose-fermenting papillae when acted on by a lysate of a mutant of the other class, but not when acted on by a lysate of a mutant of its own class.

To seek such interactions, nine O strains of *Sal. typhimurium* were selected, all of which had been isolated from natural sources as O forms; where several O strains had been isolated at the same place and time, only one from each set was included in the experiment. Lysates having been made from all of the strains which were sensitive to phage 22, strains and lysates were tested in all possible combinations, and plated on semi-solid agar; sterility controls on the lysates, and control plates of untreated recipient cells were included in the tests, and the H antigens of all swarms obtained were checked. Combinations not yielding swarms at the first attempt were tested again, using treated cells concentrated by centrifugation.

Table 4. *Action of lysates of* Sal. typhimurium *O strains on* Sal. typhimurium *O strains*

Cells of *Sal. typhimurium* O strain	Lysate of *Sal. typhimurium* O strain SW 541	SW 544	SW 545	SW 548	SW 549	SW 573	SL 15	SL 18	SL 20	Lysate of *Sal. typhimurium* H strain TM2	Control (no lysate)
SW 541	0	+	–	–	+	+	+	+	+	+	0
SW 544	S	0	–	–	S	S	S	S	S	S	0
SW 545	+	+	–	–	+	+	+	+	+	+	0
SW 548	+	+ & S	–	–	+	+	+	+	+	+	0
SW 549	+	+	–	–	0(+)	+	+	+	+	+	0(+)
SW 573	0	0	–	–	0	0	0	0	0	0	0
SL 15	+	+	–	–	+	+	0	0	0	+	0
SL 18	+	+	–	–	+	+	0	0	0	+	0
SL 20	+	+	–	–	+	+	0	0	0	+	0

+, rapid-spreading swarms; S, slow-spreading swarms; 0, no swarms; 0(+), swarms after long incubation only; –, test not made because lysate not available.

The results of this experiment are shown in Table 4. Seven of the strains fall into five groups, one group comprising three strains, the others one strain each, strains within a group being inactive on each other but active on strains of all other groups. The remaining two strains, SW 545 and SW 548, do not fall into any of the five groups defined above; they cannot be tested for action on each other, since neither can be lysed by phage 22. Where pairs of strains could be tested for interaction in each direction, the reciprocal tests gave consistent results, except that strain SW 573 was unaffected by lysates of strains which themselves responded to a lysate of strain SW 573. However, strain SW 573 also fails to respond to treatment with lysates of H strains, so that its unresponsiveness to treatment with O strains was to be expected. A possible explanation for its behaviour is proposed later.

This experiment shows that there are at least six different classes of

O strains of *Sal. typhimurium*, and therefore that there are at least six genetic factors, mutation of any one of which may cause loss of flagella. The existence of so many different genes all apparently concerned in the production of flagella may seem surprising, but there are many parallels in the genetics of other organisms. One may instance seven loci in *Bact. coli* strain K12, mutation at any one of which may result in failure to ferment lactose (Lederberg *et al.* 1951), or the seventy-three factors controlling chloroplast development in maize cited by de Haan (1933).

Strains SL15, SL18 and SL20 failed to interact in any of their possible combinations, and two *Sal. typhimurium* O strains received later (SL47 and SL48), also fall into the same group. All five strains were isolated from cases of human infection in various parts of England during 1952. These five O strains might represent repeated isolations of a single O strain widely distributed in England during 1952, or they might have resulted from independently occurring mutations affecting homologous genes in different H strains. It was therefore of interest to investigate the strains by other techniques which might tend to indicate or exclude a common origin. The results of antigenic analysis by Dr Taylor were available, and the strains were also submitted to Dr Felix for phage-typing, and to Brig. Boyd for identification of the phages (if any) carried by them (Boyd, Parker & Mair, 1951). The phage-typing results are compatible with a recent common origin, for all five strains gave the reactions of *Sal. typhimurium* Type 2*a*. The O antigens and the lysogenicity findings divide the strains into two groups. Three strains have antigen V (antigen B Vi of Felix, 1952*b*), and carry phages A2 and B2; the other two strains do not possess antigen V (in amounts detectable by agglutination tests) and carry phage B2 only. This evidence is not unequivocal, for the isolation of both V-positive and V-negative strains from the same patient (Kauffmann, 1934) suggests that antigen V may appear or disappear by mutation; and strains may be made lysogenic for phage A2 in the laboratory, and presumably therefore this may also occur in nature. It is therefore uncertain whether all the O strains of this group derived from a common O ancestor, or whether they have arisen on two or more occasions by mutations perhaps affecting the same gene.

The high frequency with which lysates of O strains render motile other O strains might suggest that the production of H forms under these conditions is not dependent on the transfer of genetic material from the lysed strain, as has been argued above, but is a consequence of exposure of an O strain to phage 22 grown on *any* heterologous host. However, phage grown on a spontaneous H mutant of O strain SW543 evoked numerous swarms from its parent O strain. Similarly, a lysate of an induced H form obtained from O strain SL15 evoked swarms from its parent O strain. In each case a lysate of the O strain itself had no effect. Tests with other O strains and their spontaneous or induced H derivatives have given similar results. Each of these H derivatives, so far as we know, differed from its O parent only by the presence of flagella, and of the gene determining this; but phage grown on the H derivative could confer motility on the O parent. Thus motility may result from exposure of an O strain to phage grown on a strain which is not an unrelated strain, provided that it possesses the gene which is deficient in the recipient O strain.

Flagellar antigenic phase latent in cells of O strains

Motile forms of *Sal. typhimurium*, obtained from O strains by transduction, underwent diphasic flagellar variation exactly as do normal H cultures of *Sal. typhimurium*. The first culture from any one swarm gave growth which was agglutinated strongly by antiserum for one of the two H antigens, weakly or not at all by antiserum for the other; that is, the swarm was (predominantly) in one phase. The phase of induced swarms was determined by the recipient O culture, and not by the inducing lysate. This was demonstrated by treating single colony broth cultures of O strains with lysates of a number of different strains. In each such experiment all or nearly all of the swarms resulting from transduction (or arising by spontaneous mutation) proved to be in one and the same flagellar phase, even when the lysate used was made from an H strain known to have been almost entirely in the opposite phase at the time of lysis. A single colony broth culture, as used in these experiments, would have been derived from one cell by a small number of generations, and would therefore be nearly homogeneous, even for a character which, like flagellar antigenic phase, commonly mutates at a very high rate (Stocker, 1949). Cells of an O strain may then be considered to be in one flagellar antigenic phase or the other, though this character is unexpressed in the absence of flagella.

Experiments on non-motile flagellated ('paralysed') strains

All motile salmonella strains have flagella, but several non-motile flagellated strains have been reported (Colquhoun & Kirkpatrick, 1932; Kauffmann, 1939; Edwards, Moran & Bruner, 1946; Hirsch, 1947; Friewer & Leifson, 1952). Hirsch's strain of *Sal. paratyphi-B* and Friewer & Leifson's strain of *Sal. typhimurium* had morphologically typical flagella, were H-agglutinable, and showed flagellar antigenic phase variation, but were non-motile under all conditions tested, as judged both by microscopy and by the absence of spreading growth in semi-solid media.

We received from Dr Leifson two non-motile H strains, labelled by us SW578 and SW580, and an O strain, our SW573; the O strain and one of the non-motile H strains (SW580) had been derived from single O and H colonies respectively, picked from the original plating of a salmonella culture submitted for identification; this primary culture also yielded colonies of normal motile flagellated *Sal. typhimurium*. The non-motile H strain SW580 had produced by mutation a motile variant; and from this motile substrain after several subcultures Leifson had selected colonies of a new non-motile H form, our strain SW578. Thus the two non-motile H strains, though derived from a common ancestor, were not necessarily genetically identical, for SW580 was isolated as a non-motile H form, whereas SW578 originated from a motile strain by mutation *in vitro*.

These two non-motile H strains when incubated on semi-solid agar at 37°

behaved exactly as did O strains; their growth was strictly confined to the site of inoculation, except for the occasional mutative production of isolated swarms. Each produced large numbers of swarms when treated with a lysate of *Sal. typhimurium* strain TM2. Thus motility can be transduced to non-motile H strains in the same way that flagellation (and resultant motility) can be transduced to O strains.

Lysates were made from both non-motile H strains; various O strains treated with either of these lysates produced swarms; this indicates that the 'paralysed' strains contain the normal homologues of the various mutant genes responsible for the absence of flagella in these O strains. Each of the non-motile H strains produced swarms when treated with a lysate of the other, but was unaffected by a lysate of itself. As in the case of the interactions of O strains, this indicates that the two strains are non-motile through different causes, i.e. mutation of non-homologous genes. The history of the strains made it clear that the lack of motility of the two strains did not result from a single mutational event, so that their genetical non-identity is less surprising than it at first seemed.

Strain SW573, the O strain isolated from the same primary culture as the non-motile H strain SW580, was the only *Sal. typhimurium* O strain studied which did not readily produce swarms when treated with lysates of other *Sal. typhimurium* strains, whether H or O. The history of this culture suggests that mutation causing loss of motility without loss of flagella occurred not infrequently in it; and a possible explanation for the failure of SW573 to produce motile swarms by transduction would be that it arose from a normal motile H strain by two successive mutations, one causing non-motility without loss of flagella, and the other causing loss of flagella. If, as seems likely, these mutations affected separate genes, the strain could only become motile by alteration of two genes, and Zinder & Lederberg (1952) found that in transduction no one cell could be shown to acquire more than a single new gene. Neither of the two forms theoretically obtainable from SW573 by transduction of a single gene would be motile. This explanation for the failure of O strain SW573 to produce swarms has been confirmed by the isolation from it of a non-motile H variant. In attempts to obtain swarms from SW573 by transduction, using lysates of various strains, repeated platings of treated cells did finally produce swarms in two instances, and in the course of these experiments a few trails were also found; on the hypothesis proposed earlier, the colonies making up a trail consist of progeny of a cell made motile by transduction, but which have themselves reverted to their original (non-motile) genetic condition. Colonies from two trails, produced by SW573 treated with lysates of O strains SW544 and SL18 respectively, were subcultured; they yielded apparently pure growth of non-motile H forms. This finding is taken to indicate that even single-colony broth cultures of strain SW573 contain a small proportion of cells which have acquired flagella by mutation, but which remain non-motile because of a 'paralysis' gene. This small fraction of mutant H cells can become motile by alteration of a single gene, and transduction affecting this small minority of mutant cells would account for the rare trails and swarms.

Experiments on slow-swarming strains

The behaviour of *Sal. typhimurium* O strain SW 544, when treated with lysates, differed from that of all the other O strains of this species which we investigated; after about 12 hr. incubation at 37°, other strains produced swarms which, when discrete, were several centimetres in diameter, whereas the swarms produced by SW 544 under similar conditions were only a few millimetres in diameter (Pl. 2, fig. 8). Subculture of these slow-spreading swarms gave colonies of motile flagellated organisms, which retained the character of spreading slowly in semi-solid medium. It is not clear why these strains spread slowly, since in broth, at room temperature or at 37°, they appear about as motile as fast-spreading strains, both as to proportion of motile cells and in rate of movement; nor do they differ from other motile strains in flagellar morphology or H-agglutinability. Strain SW 544 sometimes produced spontaneous swarms; these also were of the slow-spreading kind. The slow-swarming character of motile derivatives of strain SW 544 was fairly stable; after 12 hr. incubation on semi-solid agar, the site of inoculation was surrounded by a border of dense growth a few millimetres wide, resulting from the slow spread of the culture as a whole; a few rapid-spreading swarms were usually also present, evidently resulting from mutation (Pl. 2, fig. 9). As these spontaneous rapid-spreading swarms were few in number, it was possible to test whether the rapid-swarming character of other strains could be transduced to the slow spreaders. A slow-spreading H mutant of strain SW 544 was treated with lysates of various H and O strains; this treatment caused the appearance of numerous fast-spreading swarms. Cells treated with lysates of the slow-spreading strain itself, or of its O parent strain, SW 544, produced no more fast-spreading swarms than did control untreated cells. It is concluded that the character of slow swarming is latent in strain SW 544, though unexpressed because of the absence of flagella; that the homologous gene present in rapid-swarming strains can be transduced by phage; and that the gene concerned with the slow- or rapid-swarming character is not the same as that which determines the lack of flagella in strain SW 544.

SW 544 is our label for strain 'Glasgow O' of Schütze (1930), also known as strain OB (Felix, 1930). Schütze isolated it from the liver of a rat inoculated 3 months before with an H strain of *Sal. typhimurium*, strain 'Glasgow H', and as its somatic antigens were the same as those of the inoculated H strain he regarded it as an O variant of strain Glasgow H. We obtained strain Glasgow H (NCTC 3048) from the National Collection of Type Cultures, where it had been deposited by Schütze in 1929, to see whether it showed the slow-spreading character found in all the motile derivatives of strain Glasgow O (SW 544). Contrary to our expectation, strain Glasgow H spread through semi-solid medium with normal rapidity. The circumstances of its isolation suggested to us that strain Glasgow O might have been an independent salmonella O strain acquired by the rat through natural infection, rather than an O mutant of strain Glasgow H, which would account for the absence of the slow-spreading character in the latter strain. This suspicion was confirmed by the results of phage-typing by Dr Felix, for strain Glasgow O gave the reactions of *Sal. typhimurium* Type 1*a*, whereas strain Glasgow H reacted as Type 1*b*. Although the parentage of strain Glasgow O (SW 544) is thus unknown, its identification as *Sal. typhimurium* is established by the flagellar antigens of its spontaneous H mutants and the H forms obtained from it by transduction, and also by the results of phage-typing.

Simultaneous transduction of two flagellar characters

In previous experiments (Zinder & Lederberg, 1952), a single exposure to a lysate never resulted in the appearance of cells which had simultaneously acquired more than one new trait, even when the lysate transduced several different characters, one at a time, to different cells of the treated suspension; that is no one cell was shown to acquire more than one of the genes determining the differences between the strains. In the present work, several examples of the simultaneous transduction of two characters have been encountered; in each case both the characters transferred together relate to bacterial motility.

The two examples first met consist in the simultaneous acquisition by an O strain of the ability to produce flagella, and of a new flagellar antigen. The strains concerned were SW 543, an O strain of *Sal. paratyphi-B*, and SL 13, an O strain of *Sal. paratyphi-A*. SW 543 was received from Dr F. Kauffmann, who had found it as one component of a culture which also contained a flagellated but otherwise similar strain with H antigen *b*; as no second phase could be obtained from the H component, the culture was considered to be one of *Sal. paratyphi-B* fixed in the specific phase. Similar monophasic H forms with flagellar antigen *b* have been obtained from the O component, SW 543, by mutation. The other O strain concerned, SL 13, was an O variant isolated by Dr Felix from an H strain of *Sal. paratyphi-A* of typical antigenic constitution, that is with only a single phase and flagellar antigen *a*.

When these O strains were treated with lysates of *Sal. typhimurium* strain TM2 most of the swarms which they produced had the H antigen characteristic of the recipient strain, that is *b* or *a* respectively; as these antigens are not found in the donor strain we conclude that these O strains retain the potentiality of producing their characteristic flagellar antigens, and this potentiality we presume is located in a gene (or genes). In addition, both strains produced a minority of swarms with flagellar antigen *i*, an antigen present in the donor strain, but not latent in the recipient O strains (so far as can be tested). These derivatives with an exogenous H antigen, like those with the intrinsic antigens *b* or *a*, were monophasic. The simplest hypothesis to account for these findings is that each of these O strains contains a mutated gene determining the absence of flagella and another gene which determines the antigenic composition of the flagella when present, and that the donor strain of *Sal. typhimurium* contains homologous genes, one of which determines the H antigenic constitution of *Sal. typhimurium* in the specific phase, that is antigen *i*. The transfer of one only of these genes would result in H cells with the flagellar antigen characteristic of the recipient strain, the transfer of both would result in H cells having antigen *i*. The genetic basis of these coupled transductions is being studied further, but two lines of evidence lend support to the above interpretation.

The two O strains SW 543 and SL 13 have been exposed to lysates of salmonellas other than *Sal. typhimurium*, and in every case when swarms were produced a minority of them had an H antigen characteristic of the lysed strain. Thus SW 543 treated with lysates of *Sal. sendai*, *Sal. altendorf* and *Sal. heidelberg* produced swarms, a minority of which were monophasic H forms with flagellar antigens *a*, *c* and *r* respectively. In each case the 'partial hybrid' had the same O antigenic constitution as the O parent strain, that is IV, V, XII. Similarly, strain SL 13 treated with a lysate of *Sal. heidelberg* produced swarms most of which had the antigenic constitution I, II, XII: *a*; -, and a few which were I, II, XII: *r*; -. Several of these antigenic combinations have not been reported in naturally occurring *Salmonella* spp., and such monophasic H forms may be of value for the production of diagnostic serological reagents (cf. Edwards & Bruner, 1946).

Sal. paratyphi B O strain SW 543, treated with a lysate of *Sal. typhimurium*, produced two kinds of swarms, *b* and *i*, and we attribute the latter kind to the simultaneous transfer of two genes. An alternative hypothesis postulates that strain SW 543 lacks flagella through the presence of a 'suppressor' gene with the property of preventing the development of flagella of antigenic type *b*, but not of flagella of other antigenic types. Transduction of the normal homologue of the suppressor gene, from *Sal. typhimurium*, would then result in *b* swarms; transduction of the determinant for H antigen would result in *i* swarms. This hypothesis requires only single gene transductions. However, it is excluded by the results of experiments on the transducing activity of lysates of the *i* swarms obtained from strain SW 543 by exposure to lysates of *Sal. typhimurium*. Such lysates provoke both *b* and *i* swarms from their parent O strain, SW 543, as would be expected on our hypothesis that the *i* swarms resulted from the simultaneous transfer of two genes. On the alternative hypothesis of a 'suppressor' gene and single gene transfers only, the *i* derivatives of strain SW 543 would retain the gene suppressing the development of *b* flagella, and lysates of them would therefore be unable to provoke *b* swarms from SW 543, which *ex hypothesi* also carries the *b* suppressor gene.

Retesting of our other O strains has shown that *Sal. dublin* O strain SW 553 also produces a minority of swarms with an H antigen characteristic of the donor strain, instead of antigen *g,p* known to be latent in SW 553. No further examples of double transduction of this kind have been found, even when they were selected for by plating O cells on semi-solid agar containing antibody for their latent H antigens, but not for the H antigens of the donor strain. It appears then that the 'O' genes which determine absence of flagella in strains SW 543, SL 13 and SW 553 are related to a gene which determines flagellar antigenic specificity, in such a way that when the 'O' gene is replaced by its normal homologue from another strain, the gene for the intrinsic H antigen (*b*, *a*, or *g,p*) is frequently also replaced by a homologous gene determining a flagellar antigen of the donor strain. Experiments intended to test the homology of the genes causing absence of flagella in strains SW 543, SL 13 and SW 553 are in progress.

What is probably a further case of simultaneous transfer of two characters was met in the course of testing O strains for interaction. *Sal. typhimurium* O strain SW 548 when it acquired motility, by mutation or through transduction from strains other than SW 544, produced only fast-spreading swarms; but when treated with a lysate of SW 544 it produced some fast and some slow swarms (Pl. 2, fig. 10). Strain SW 544 (Glasgow O) though it lacks flagella is known to carry the slow-swarming character, and the slow-swarming H forms obtained from SW 548 by treatment with lysates of SW 544 resembled the slow-swarmers which the latter strain produces when it acquires the H character by mutation or transduction. The fast-spreading swarms obtained from O strain SW 548 by the action of lysates of SW 544 may be explained by the transfer from SW 544 of a single gene, i.e. the normal homologue of the gene determining the O state of SW 548. The slow swarms produced by the same treatment we think result from the simultaneous acquisition by some cells of a second gene, the 'slow-swarming' gene of strain SW 544. Our investigation of this case has been limited by our inability to test the action of lysates of SW 548 and its H derivatives, for they are all resistant to phage 22. An alternative but perhaps less probable explanation involving a 'suppressor' gene therefore cannot be tested. No other O strain has been observed to acquire the slow-swarming character when it acquires motility by treatment with a lysate of strain SW 544. On the simultaneous transduction hypothesis it appears that the gene determining the absence of flagella in strain SW 548 is related to a gene which determines slow or rapid spreading in such a way that when one is replaced by transduction so often is the other; but that the (non-homologous) genes determining absence of flagella in the other O strains are not so related to the 'speed of swarming' gene.

DISCUSSION

Genetics of flagellar characters in salmonellas

The experiments described show that various flagellar character may be conferred on salmonella strains by transduction from other strains; flagellation has been transduced to O strains, motility to non-motile H strains, and the rapid-swarming character to slow-swarming strains. Since in general only one character has been transferred at a time the transducing activity of lysates must be localized in material particles of different kinds, i.e. having different genetic effects; and these particles must have come from the cells which were lysed. As the characters conferred are retained on subculture, the material which confers a new character must be replicated and transmitted to all the progeny of the cell which acquires it. Thus these experiments support the hypothesis that there is in salmonellas specialized material determining, in part at least, the hereditary properties of the organism, and that this material is divisible into qualitatively different localized units concerned with the control of different characters; that is, the hypothesis that salmonellas, like other organisms, have genes.

The experiments on transduction of flagellation to O strains by treatment with lysates of other O strains show the existence of a number of different genes, mutation of any one of which may cause absence of flagella. Latent H antigens were demonstrated in all the O strains tested. One may compare the retention of latent H antigens by O strains with the behaviour of H strains grown under conditions such that flagella are not formed, e.g. on phenol agar. In each case cells without flagella retain and transmit the ability to form a particular H antigen when flagella are produced, i.e. after mutation or transduction of the H character in the one case, or on transfer to a suitable medium in the other. On the gene theory this ability is localized in a gene or genes regulating H antigen specificity.

The experiments on non-motile H strains and on slow-swarming strains show the existence of several genes concerned with flagellar function. So far as they have been investigated, these genes seem to be different from each other, from the genes determining H antigenic specificity, and from those determining the presence or absence of flagella. Lewin (1952) has reported comparable genes controlling flagellar function in the green alga *Chlamydomonas moewusii*.

The experiments so far discussed indicate the existence of a number of different genes concerned with various flagellar characters, but do not indicate how these genes are arranged in the cell. Some inferences as to this may be drawn from the exceptional instances of simultaneous transduction of two characters. In each instance there is a relationship between the members of a particular pair of genes such that cells which acquire one of the pair by transduction frequently acquire the other also. If in salmonellas the genes are arranged in a constant ordered array, it may be that in transduction genes which are close together in the array will sometimes be carried over together in a single fragment of the gene array. On this hypothesis the genes which can be simultaneously transduced would be genes adjacent to each other in the array. In all organisms whose genetics have been extensively investigated the genes behave as if arranged in linear groups, which correspond to microscopically demonstrable chromosomes; one may suppose that in salmonellas also the postulated gene array consists of one or more linear groups.

It is noteworthy that in each of the instances of simultaneous transduction so far observed, both the characters transferred together have related to the same organelle, i.e. flagella. Experiments intended to reveal the simultaneous transduction of pairs of unrelated metabolic characters (Zinder & Lederberg, 1952), or of a metabolic character (xylose or galactose fermentation) and of flagellation to an O strain, have given negative results. One may speculate on the possibility that pairs of salmonella genes which can be transduced together correspond to the groups of very closely linked loci all concerned with the same character or function which are familiar in the genetics of other organisms, e.g. the closely linked Lac_{1a}, Lac_{1b} and Lac_4 loci of *Bact. coli* K12, mutation at any of which leads to failure to ferment lactose (Lederberg, 1952).

The phenomenon of transduction

All the experiments here described have been compatible with the hypothesis (Zinder & Lederberg, 1952) that transduction of characters in salmonellas results from transference of genetic material by phage particles from the cells in which they were formed to cells which absorb the phage. Some new observations on the process have been made. The detection of some motile but non-lysogenic cells in induced swarms has shown that phage is not always transmitted to all the progeny of cells showing a transduced character. This divorce of transducing phage from transduced character has also been seen in the transduction of other characters, e.g. the ability to ferment galactose. The trail phenomenon in the transduction of motility suggests that phage-imported genetic material which modifies the character of the cell containing it sometimes fails to replace the homologous genetic material of the recipient cell, and therefore fails to be replicated. The simultaneous transduction of two characters to a single cell has been demonstrated for the first time. In each instance observed, both characters have related to flagella, but have been shown to be determined by separate genes. This suggests that in transduction the genetic material carried over consists not of units of the smallest possible size, but of small fragments of a genetic structure, perhaps short pieces of chromosome.

The present work emphasizes the relationship between transduction of characters in *Salmonella* spp. and the phenomena of genetic transfer in pneumococcus and *H. influenzae* termed 'transformation'. The vector of transduction in salmonellas appears to be a phage particle, whereas the agent concerned in the other genera has the properties of undepolymerized deoxyribonucleic acid; but genetically the phenomena are very similar. In both systems the characters which the active agent can confer are determined by the hereditary properties of the strain from which it was obtained. The production of flagellated forms by salmonella O strains treated with lysates of other O strains is formally similar to what has been termed 'allogenic transformation' of pneumococci, that is the production by a transformation reaction of a pneumococcal strain showing a character (presence of capsules of normal size) not shown by either the recipient strain or the donor strain from which the transforming agent was extracted (Ephrussi-Taylor, 1951*a*, *b*); the explanation proposed for allogenic transformation by Ephrussi-Taylor is not essentially different from that suggested for the analogous phenomenon in salmonellas, namely, that the character is only expressed when two different genetic factors are both present together in the cell.

A difference between comparable cases of transference of characters in the two groups is the retention of (latent) flagellar antigenic specificity by all non-flagellated salmonellas mutants, and the apparent absence of latent capsular antigenic specificity in some non-encapsulated mutants of pneumococci and *H. influenzae*; for the latter when transformed to encapsulation by transforming agent obtained from heterologous strains produce capsules whose antigenic specificity is that of the donor strain. We take this to indicate that the strains in question became non-encapsulated either by mutation of that gene which determines capsular antigenic specificity, or by mutation of a second gene so closely linked to the first that in transformation reactions both genes travel together. However, Griffith (1928) observed non-encapsulated pneumococcal mutants which might either regain capsules of the antigenic type of their ancestors, or acquire capsules of the antigenic type of the donor strain, a situation analogous to that which we have found in certain O strains which when they acquire flagellation by transduction sometimes but not always acquire also an H antigen from the donor strain.

In both groups it is in general only possible to transfer one character at a time to any individual cell. The simultaneous transduction of two flagellar characters in salmonellas reported above has, however, a parallel in the simultaneous transference of two separable capsular antigenic characters in *H. influenzae* (Leidy, Hahn & Alexander, 1953). Certain observations on allogenic transformation in type III pneumococci led Ephrussi-Taylor (1951 *a*, *b*) to postulate that the genetic agent involved in 'type transformation' was spatially differentiated, so that either the whole agent might be transferred, or one part of it only might replace the homologous part in a corresponding agent present in the recipient cell.

Thus the genetic aspects of the phenomena in *Salmonella* spp., and in pneumococci and *H. influenzae* resemble one another even in the exceptional occurrence of simultaneous transfer of two characters. There seem to be no experimental data incompatible with the view that the genetic particles concerned in the two groups are of the same kind, and that the differences between the phenomena result from the fact that in salmonellas the fragments of genetic material can only be effectively transferred from cell to cell by their association with phage particles.

The work of one of the authors (B.A.D.S.) was in part undertaken during the tenure of a Commonwealth Fund Fellowship. The work at Madison has been supported by grants (E72–C4) from the National Microbiological Institute of the National Institute of Health, U.S. Public Health Service, from the Rockefeller Foundation and from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. This paper is recorded as no. 516 from the Department of Genetics, University of Wisconsin.

We are indebted to Dr P. R. Edwards and Dr J. Taylor for the serological examination of many strains, to Dr A. Felix, F.R.S., and to Dr E. S. Anderson for phage-typing, to Brig. J. S. K. Boyd, F.R.S., for determinations of lysogenicity, and to them and Dr F. Kauffmann, Dr N. A. Boulgakov, Dr K. Lilleengen, Dr E. Leifson, Dr S. T. Cowan, Lt.-Col. H. J. Bensted and Dr N. Datta for the generous provision of cultures, phages and sera.

REFERENCES

Alexander, H. E. & Leidy, G. (1951). Determination of inherited traits of *H. influenzae* by desoxyribonucleic acid fractions isolated from type-specific cells. *J. exp. Med.* **93**, 345.

Andrewes, F. W. (1922). Studies in group-agglutination. I. The Salmonella group and its antigenic structure. *J. Path. Bact.* **25**, 505.

Andrewes, F. W. (1925). Studies in group agglutination. II. The absorption of agglutinin in the diphasic salmonellas. *J. Path. Bact.* **28**, 345.

Avery, O. T., MacLeod, C. M. & McCarty, M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *J. exp. Med.* **79**, 137.

Boyd, J. S. K. (1950). The symbiotic bacteriophages of *Salmonella typhi-murium*. *J. Path. Bact.* **62**, 501.

Boyd, J. S. K. (1951). 'Excessive dose' phenomenon in virus infections. *Nature, Lond.* **167**, 1061.

Boyd, J. S. K., Parker, M. T. & Mair, N. S. (1951). Symbiotic bacteriophage as a 'Marker' in the identification of strains of *Salmonella typhi-murium*. *J. Hyg., Camb.* **49**, 442.

Braun, H. (1918). Das Wesen der Weil-Felix'schen Reaktion auf Fleckfieber. *Berl. klin. Wschr.* **55**, 637.

Colquhoun, D. B. & Kirkpatrick, J. (1932). The isolation of motile organisms from apparently non-motile cultures of *B. typhosus*, *B. proteus*, *B. pestis*, *B. melitensis*, etc. *J. Path. Bact.* **35**, 367.

de Haan, H. (1933). Inheritance of chlorophyll deficiencies. *Bibliogr. genet.* **10**, 357.

Edwards, P. R. & Bruner, D. W. (1942). Serological identification of Salmonella cultures. *Circ. Ky agric. Exp. Sta.* no. 54.

Edwards, P. R. & Bruner, D. W. (1946). Notes on monophasic Salmonella cultures and their use in the production of diagnostic serums. *J. Bact.* **52**, 493.

Edwards, P. R., Moran, A. B. & Bruner, D. W. (1946). Flagella and flagellar antigens in 'non-motile' *Salmonella* cultures. *Proc. Soc. exp. Biol., N.Y.*, **62**, 296.

Ephrussi-Taylor, H. (1951*a*). Transformations allogènes du pneumocoque. *Exp. Cell Res.* **2**, 589.

Ephrussi-Taylor, H. (1951*b*). Genetic aspects of transformations of pneumococci. *Cold Spr. Harb. Symp. quant. Biol.* **16**, 445.

Felix, A. (1930). The qualitative serum diagnosis of enteric fevers. *Lancet*, i, 505.

Felix, A. (1951). Laboratory control of the enteric fevers. *Brit. med. Bull.* **7**, 153.

Felix, A. (1952*a*). The Vi antigen of *Salmonella paratyphi-A*. *J. Hyg., Camb.* **50**, 540.

Felix, A. (1952*b*). The Vi antigen of *Salmonella paratyphi-B*. *J. Hyg., Camb.* **50**, 550.

Felix, A. & Callow, B. R. (1943). Typing of paratyphoid-B bacilli by means of Vi bacteriophage. *Brit. med. J.* ii, 127.

Felix, A. & Callow, B. R. (1951). Paratyphoid-B Vi-phage Typing. *Lancet*, ii, 10.

Felix, A. & Pitt, R. M. (1951). The pathogenic and immunogenic activities of *Salmonella typhi* in relation to its antigenic constituents. *J. Hyg., Camb.* **49**, 92.

Friewer, F. I. & Leifson, E. (1952). Non-motile flagellated variants of *Salmonella typhi-murium*. *J. Path. Bact.* **64**, 223.

Griffith, F. (1928). The significance of pneumococcal types. *J. Hyg., Camb.* **27**, 113.

Hirsch, W. (1947). A new bacterial variant: the non-motile H form. *J. Hyg., Camb.* **45**, 417.

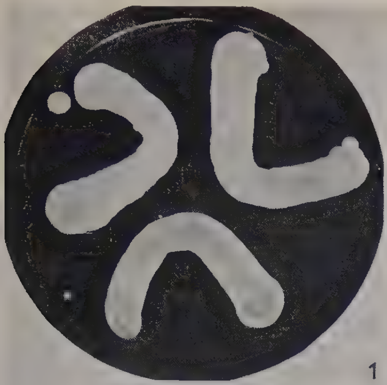
Kauffmann, F. (1934). Über serologische und kulturelle Variant Paratyphus-B und Mäusetyphus-Bacillen. *Z. Hyg. Infekt-Kr.* **116**, 368.

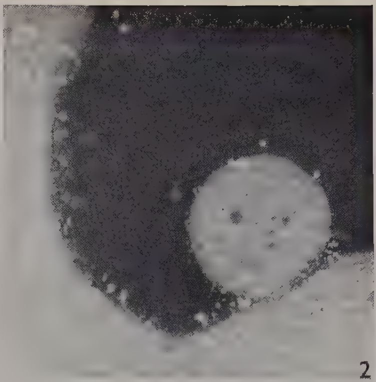
Kauffmann, F. (1939). Die serologische Salmonella-diagnose. *Acta path. microbiol. scand.* **16**, 417.

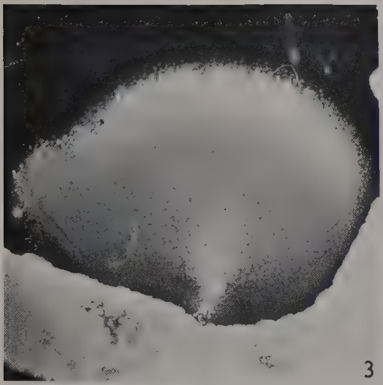
Kauffmann, F. (1947). On the serology of the *Salmonella* V antigen. *Acta path. microbiol. scand.* **24**, 591.

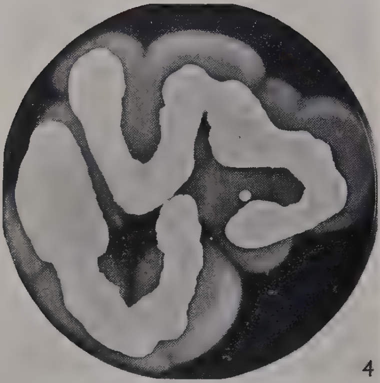
Kauffmann, F. (1951). *Enterobacteriaceae.* Copenhagen: Einar Munksgaard.

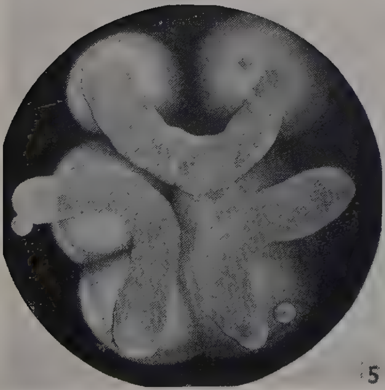
Lederberg, E. M. (1952). Allelic relationships and reverse mutation in *Escherichia coli*. *Genetics*, **37**, 649.

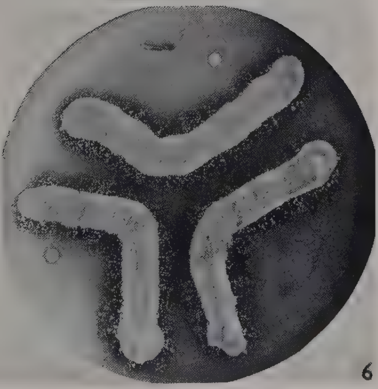

1


2


3

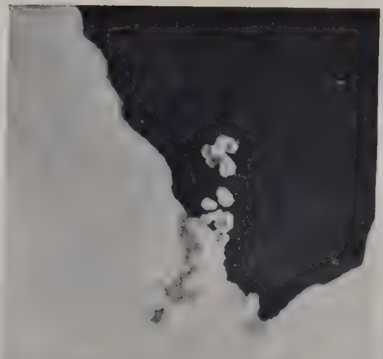

4


5

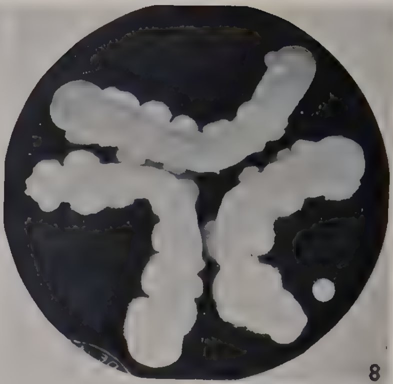

6

B. A. D. STOCKER *et al.*—TRANSDUCTION OF FLAGELLAR CHARACTERS. PLATE 1

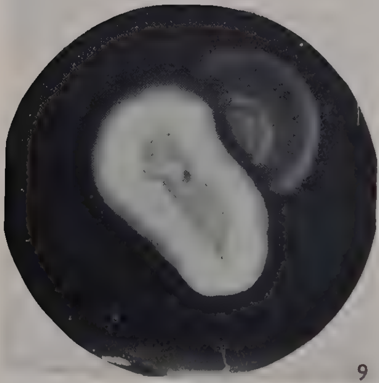
Journal of General Microbiology, Vol. 9, No. 3



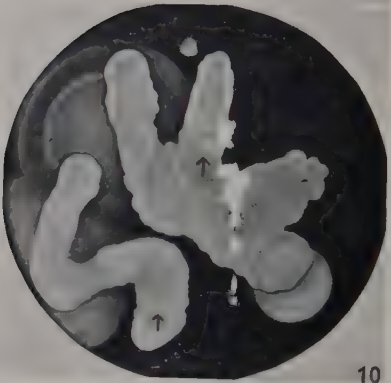
7



8



9



10

B. A. D. Stocker *et al.* —Transduction of flagellar characters. Plate 2

Lederberg, J., Lederberg, E. M., Zinder, N. D. & Lively, E. R. (1951). Recombination analysis of bacterial heredity. *Cold Spr. Harb. Symp. quant. Biol.* **16**, 413.

Leidy, G., Hahn, E. & Alexander, H. E. (1953). *In vitro* production of new types of *Haemophilus influenzae. J. exp. Med.* **97**, 467.

Lewin, R. A. (1952). Ultraviolet induced mutations in *Chlamydomonas moewusii* Gerloff. *J. gen. Microbiol*, **6**, 233.

Lilleengen, K. (1948). Typing of *Salmonella typhi-murium* by means of bacteriophage. *Acta path. microbiol. scand.* Suppl. 77.

Lwoff, A. (1953). The nature of phage reproduction. In *The Nature of Virus Multiplication. Symp. Soc. gen. Microbiol.* 1952. p. 149.

Salmonella Sub-Committee (1949). Third report of the Salmonella sub-committee of the Nomenclature Committee. *Congr. int. Microbiol.* (Copenhagen, 1947), p. 617.

Schütze, H. (1930). The importance of somatic antigen in the production of Aertrycke and Gaertner immunity in mice. *Brit. J. exp. Path.* **11**, 34.

Sertic, V. & Boulgakov, N. A. (1936). Bactériophages specifiques pour des variétes bactériennes flagellées. *C.R. Soc. Biol., Paris*, **123**, 887.

Stocker, B. A. D. (1949). Measurements of rate of mutation of flagellar antigenic phase in *Salmonella typhimurium. J. Hyg., Camb.* **47**, 398.

Zinder, N. D. & Lederberg, J. (1952). Genetic exchange in *Salmonella. J. Bact.* **64**, 679.

EXPLANATION OF PLATES

Cultures on semi-solid medium in 5 cm. diameter Petri dishes, photographed by oblique transmitted light. To enhance the visibility of swarms, the plates after primary incubation at 37° have been re-incubated at 23°; at this temperature growth continues, but further spreading is prevented by solidification of the medium.

Plate 1

Fig. 1. Growth confined to sites of inoculation. O strain SW548, untreated, incubated at 37° for 48 hr. ×1.

Fig. 2. O strain SW545, untreated, incubated at 37° for 18 hr., showing discrete colonies at a distance from site of inoculation. ×5.

Fig. 3. Swarm with flare; non-motile H strain SW578 treated with a lysate of strain SW580 and incubated at 37° for 10 hr. (The appearance of discrete colonies at the periphery of the swarm is an artifact resulting from secondary incubation.) ×5.

Fig. 4. Continuous border of swarming; O strain SW548 treated with a lysate of strain TM2 and incubated at 37° for 10 hr. ×1.

Fig. 5. Discrete swarms; as Fig. 4 but treated cells diluted 1 in 10 before inoculation. ×1.

Fig. 6. Confluent swarms and trails; O strain SW541 treated with a lysate of strain TM2 and incubated at 37° for 18 hr. ×1.

Plate 2

Fig. 7. A single trail; as Fig. 6, but treated cells diluted 1 in 30 before inoculation. ×5.

Fig. 8. Slow-spreading swarms; strain SW544 treated with a lysate of strain TM2 and incubated at 37° for 10 hr. ×1.

Fig. 9. Slow-spreading mutant of strain SW544, untreated, incubated at 37° for 10 hr. One rapid-spreading (mutative) swarm is visible. ×1.

Fig. 10. O strain SW548 treated with a lysate of strain SW544 and incubated at 37° for 10 hr. Three fast-spreading and three slow-spreading swarms (indicated by arrows) are visible. ×1.

(*Received* 18 *May* 1953)

Conner, R. L., van Wagtendonk, W. J. & Miller, C. A. (1953). *J. gen. Microbiol.* 9, 434–439.

The Isolation from Lemon Juice of a Growth Factor of Steroid Nature required for the Growth of a Strain of *Paramecium aurelia*

By R. L. CONNER,* W. J. van WAGTENDONK
and C. A. MILLER

Department of Zoology, Indiana University, Bloomington, Indiana, U.S.A.

SUMMARY: A steroid fraction obtained from lemon juice was found to be an essential metabolite for *Paramecium aurelia*, var. 4, stock 51.7 (s), in axenic culture. This steroid is closely related to, or is identical with, either β-sitosterol or γ-sitosterol.

Paramecium aurelia has been established in pure culture in a heat-sterilized medium, composed of equal parts of a 0·5 % yeast autolysate (Basamin-Busch) and a 24 hr. culture of *Aerobacter aerogenes* in lettuce extract (van Wagtendonk & Hackett, 1949). Lettuce or other plant extracts were shown to be essential since no growth of *P. aurelia* could be obtained when these extracts were omitted. An axenic medium, consisting of a salt solution, dialysed yeast extract, a solution of water-soluble vitamins, proteose peptone, and guanylic and cytidilic acid has been developed (van Wagtendonk, Miller & Conner, 1952; van Wagtendonk, Conner, Miller & Rao, 1953). *P. aurelia* is unable to grow in this medium unless plant extracts are added. An essential metabolite for *P. aurelia* must therefore be present in plants. The isolation and identification of this metabolite is described.

EXPERIMENTAL

Method of assay

P. aurelia, variety 4, stock 51.7, sensitive (Sonneborn, 1949) is not able to grow in either axenic or bacterized media in the absence of plant extracts. Rapid screening assays for activity are desirable in an extraction of biologically active material. The test in axenic medium is slow, and for this reason fractions were screened using a bacterized medium. The basal medium for the bacterial assay consisted of: NaCl, 0·3 g.; KCl, 0·05 g.; Na_2HPO_4, 0·1 g.; NaH_2PO_4, 0·1 g.; $CaCl_2.2H_2O$, 0·05 g.; $MgSO_4.7H_2O$, 0·01 g.; $FeSO_4(NH_4)_2SO_4.24H_2O$, 0·125 g.; double distilled water, 1 l.; HCl to pH 6·5.

The extracts to be tested were prepared in serial dilutions, using the salt solution as a diluent. These dilutions were autoclaved at 120° for 15 min. They were then inoculated with *A. aerogenes* 24 hr. before use for the assay, as in the procedure routinely used for the culture of *P. aurelia* (Sonneborn, 1950).

---

* Predoctoral Fellow of the National Science Foundation.

One-half ml. of each dilution of the fractions being tested was placed in each depression of a 3-depression slide. Single isolations of *P. aurelia* were placed in each depression, and the depressions incubated at 27°. After 24 hr. the number of animals present in each depression was counted and a single isolation from each depression was made into fresh culture fluid of the same composition. This was repeated three times. In each series a control, consisting of the basal medium with *A. aerogenes* alone, was included. No multiplication of *P. aurelia* took place in these controls, except during the first 24 hr.

In order to express quantitatively the activities of the different fractions as measured in the bacterized medium, it was necessary to adopt a unit of activity. One unit was arbitrarily defined as follows: a solution of an active fraction contains one unit when *P. aurelia*, var. 4, stock 51.7 (s), transferred to the medium, will undergo at least three fissions per day on three consecutive transfers of a single animal.

The highly purified fractions were tested qualitatively in an axenic medium prepared as follows: One pound of Fleischmann's bakers' yeast was finely crumbled and dried at 65°. A 1 : 4 (w/v) suspension of the dried yeast was prepared by continuously stirring the yeast in distilled water at 60° for 3 hr. The suspension was autoclaved for 20 min. at 120°, centrifuged, and filtered through a Seitz filter pad (no. 8) for sterilization and clarification. A 1% solution of proteose peptone (Difco) was made in distilled water and autoclaved for 20 min. at 120°. One volume of the yeast extract was added aseptically to 5 vol. of the proteose peptone solution. In this medium the factor from plant extracts is the limiting component. The fractions were made up in double distilled water in the optimal concentration as determined by the bacterial assay method. Such a fraction was considered to be active when *P. aurelia*, transferred to this medium, showed sustained growth for at least four consecutive transfers. This assay served to eliminate the possibility that the factor from lemon juice was altered by the bacteria and thereby made available to *P. aurelia.*

After the factor had been obtained in the crystalline state and identified as a steroid, direct comparisons between the population densities obtained with other steroids were possible. Serial dilutions of the factor and of the steroids were made in the following manner: a weighed amount was dissolved in 95% ethanol, so that the resulting solution contained 1 mg./ml. This solution was then added by means of a fine pipette to double distilled water heated to 70°; this resulted in the formation of a stable suspension. The final concentration of all compounds so prepared was 100 μg./ml. Further serial dilutions were made with distilled water. The tubes were then autoclaved. Desired amounts of these dilutions were added aseptically to the axenic medium described above. Approximately thirty animals were inoculated into each tube and incubated for 4 days at 27°. After four such transfers the number of animals/ml. in each tube was determined by counting the number of organisms present in suitable samples. (Nephelometric techniques could not be applied because of intense light scattering by the organisms.) Except for the first transfer, no multiplication of *P. aurelia* took place in the absence of the factor from lemon juice. All concentrations were tested in duplicate and every assay was repeated at least three times.

Distribution of the growth factor

Various plant and animal sources were tested for growth-promoting activity. The results are given in Table 1. No preparations from animal sources were active. It is not known whether the activity displayed by the different plant extracts is due to the same factor. Lemon concentrate was the most active source and was selected for the extraction and purification of the growth factor.

Table 1. *Relative activity of various raw materials*

	Relative activity
Cerophyl extract*	1
Water extract of desiccated lettuce (Difco)	3
Water extract of baked lettuce	4
Orange juice (fresh)	10
Lemon juice (fresh)	15
Lemon concentrate†	60
Lemon peel infusion†	0
Wilson liver extract	0
Proteose peptone	0
Bacto tryptone	0
Casamino acids	0

* Obtained from the Cerophyl Laboratories through the courtesy of Dr G. A. Köhler.

† Obtained from the California Fruit Growers Exchange through the courtesy of Dr W. E. Baier.

Extraction and purification of the growth factor from lemon juice

The extraction procedure included the following steps: (1) precipitation of the active fraction with acetone; (2) extraction of the precipitate with hot ethanol; (3) saponification of the extract with methanolic KOH; (4) phase distribution; (5) chromatography. Steps 1–4 are given in Table 2, and step 5 in Table 3. In the first two steps, the precipitation and the extraction of the precipitation with hot ethanol, the total activity increased, presumably caused by the removal of inhibitory substances. The saponification of the ethanolic extract resulted in a large increase in potency/μg., as does the following phase distribution.

Three distinct bands appear in the chromatogram (Table 3). Most of the activity is present in the second band. After elution with hot ethanol a yellow solution was obtained from which white crystals separated after cooling to $-10°$. The inactive yellow material was removed by repeated recrystallization from 95% ethanol at 4°. A pure compound, representing 0·025% of the original solids and 20% of the original activity, was isolated. The chemical characteristics of this compound are given in Table 4.

The infra-red spectrum of the growth factor for *P. aurelia* is very similar to that of a sample of β-sitosterol (Fig. 1).

Table 2. *Precipitation, extraction, and phase distribution steps for the purification of the growth factor for* Paramecium aurelia, *var.* 4, *stock* 51.7 (*s*)

The yields and activities are based on assays with the bacterized medium.

Treatment	Fraction	Solids (%)	Activity (%)	Potency units/μg.
1 gal. of lemon concentrate, chill to 4° and add equal volume of cold acetone, stir well and centrifuge at 1000*g* at 4° (supernatant solution, inactive)	1	100	100	0·125
Precipitate	2	37	150	0·5
Suspend P_1 in hot 95% ethanol (500 ml.), stir, filter while hot. Repeat 3 times, combine extract (residue inactive) and concentrate to solids	3	7	170	8
Dissolve E in 500 ml. of hot 80% methanolic KOH (10%). Allow to stand for 4 hr. with occasional shaking. Extract 3 times with 200 ml. petroleum ether (30–60°). Combine petroleum ether extracts and concentrate to solids	4	0·6	96	20
Methanol layer of E	5	6·4	3	0·5
Dissolve R in 100 ml. 80% methanol and extract 3 times with CCl_4. Dry with anhydrous $MgSO_4$, filter and concentrate *in vacuo*	6	0·3	96	40
Methanol layer of R	7	0·3	2	10

Table 3. *Alumina column step in the purification for the growth factor for* Paramecium aurelia, *var.* 4, *stock* 51.7 (*s*)

4·5 g. of fraction 6 (Table 2) was dissolved in 25 ml. CCl_4. 5 ml. portions were added to columns (1·5 × 10 cm.) of 12 g. cyanide-treated, acetic-acid-washed alumina (Eli Lilly and Co., Indianapolis, Indiana. Lot number 190–4B–292). The column was washed with 25 ml. CCl_4 and developed with CCl_4-ethyl ether (9·1) till 3 bands separated. A total of 25 ml. of the developer was used. The column was cut in three portions corresponding to the bands, and each portion was eluted with hot ethanol. The combined fractions of five such chromatograms were combined and evaporated *in vacuo* to solids.

Band no.	Colour	Fraction	Solids (%)	Activity (%)	Potency units/μg.
1	Bright yellow	D_1	0·05	0	0
2	Deep orange	D_2	0·20	107	67
3	Light yellow	D_3	0·05	0·4	10

Table 4. *Chemical characteristics of the factor from lemon juice*

Melting point 136·5–137·5° (uncorrected).
Carbon and hydrogen analysis.

	C	H
Lemon factor	83·60	12·32
Calculated for: $C_{27}H_{46}O$	83·80	11·91
$C_{29}H_{48}O$	84·46	11·65
$C_{29}H_{50}O$	83·92	12·07

Colour tests

Liebermann-Burchard +. Salkowski +. Rosenheim −.

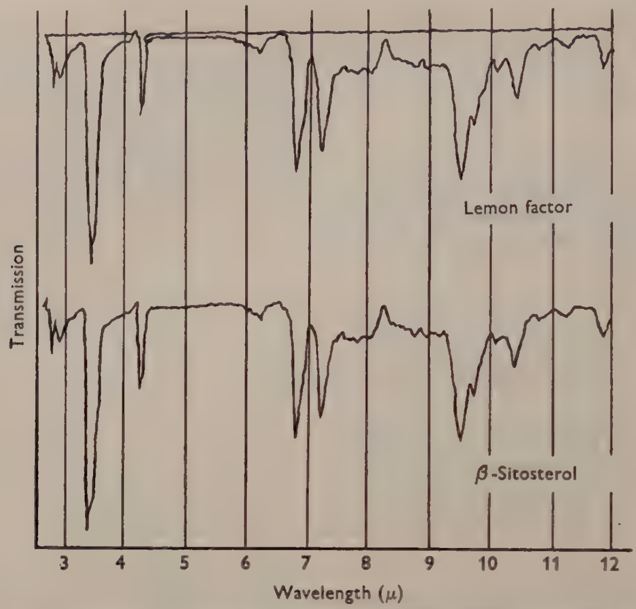


Fig. 1. Infra-red adsorption curves of the steroid from lemon juice and of β-sitosterol. The curves are tracings from original automatic recordings. Second curve: steroid from lemon juice (Paramecium factor), 11·0 % in chloroform. Third curve: β-sitosterol, 11·3 % in chloroform. Chloroform (first curve) was passed over SiO_2 and Al_2O_3 to remove traces of water and alcohol.

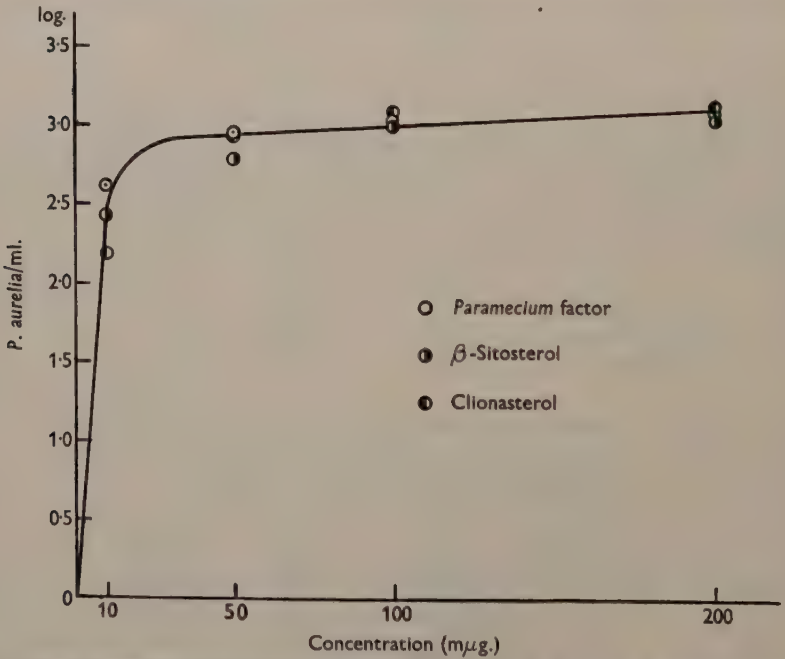


Fig. 2. Growth response of *Paramecium aurelia*, var. 4, stock 51.7 (*s*), in axenic culture to increasing concentrations of the steroid, isolated from lemon juice, β-sitosterol and clionasterol.

The growth-promoting activity of the factor from lemon juice was compared with that of β-sitosterol and clionasterol (inferred to be the 24*a*-epimer of β-sitosterol (Bergmann & Low, 1947; Fieser & Fieser, 1949)). It can be seen from Fig. 2, that the three compounds have very similar growth-promoting activities.

Because of (1) the close similarity of the infra-red spectra of the factor from lemon juice and β-sitosterol, and (2) the fact that the factor from lemon juice, β-sitosterol and clionasterol have similar growth-promoting activities, it is tentatively concluded that the growth factor for *P. aurelia*, var. 4, stock 51.7 (s), is closely related to, or identical with, either β- or γ-sitosterol. The possibility is not excluded that the fraction isolated from lemon juice is a mixture of these two isomers.

We wish to thank Dr W. E. Baier (California Fruit Growers Exchange) for generous supplies of lemon concentrate, Dr W. Bergmann for samples of various steroids, and Dr A. van Abeele and Dr H. Boaz (Eli Lilly and Co.) for their co-operation in performing the C, H analyses and infra-red adsorption spectra. This work was carried out under a contract between Indiana University and the Office of Naval Research (Contract no. 60 nr-18010). It was also supported by grants from the Rockefeller Foundation and Indiana University.

This paper is contribution no. 478 from the Department of Zoology, Indiana University.

REFERENCES

BERGMANN, W. & LOW, E. M. (1947). Contributions to the study of marine products. XX. Remarks concerning the structure of sterols from marine invertebrates. *J. org. Chem.* 67.

FIESER, L. F. & FIESER, M. (1949). *Natural Products Related to Phenanthrene*, 3rd ed. New York: Reinhold Publishing Corporation.

SONNEBORN, T. M. (1949). Beyond the gene. *Amer. Scient.* **37**, 33.

SONNEBORN, T. M. (1950). Methods in the general biology and genetics of *Paramecium aurelia*. *J. exp. Zool.* **113**, 87.

WAGTENDONK, W. J. VAN & HACKETT, P. L. (1949). The culture of *Paramecium aurelia* in the absence of other living organisms. *Proc. nat. Acad. Sci., Wash.* **35**, 155.

WAGTENDONK, W. J. VAN, MILLER, C. A. & CONNER, R. L. (1952). Growth requirements of *Paramecium aurelia*, var. 4, stock 51.7 (s), in a medium free of other living organisms. *Fed. Proc.* **11**, 302.

WAGTENDONK, W. J. VAN, CONNER, R. L., MILLER, C. A. & RAO, M. R. R. (1953). Growth requirements of *Paramecium aurelia*, var. 4, stock 51, sensitives and killers, in axenic medium. *Ann. N.Y. Acad. Sci.* (in the Press).

(*Received* 18 *May* 1953)

Postgate, J. R. (1953). *J. gen. Microbiol.* **9**, 440–444.

On the Nutrition of *Desulphovibrio desulphuricans*: a Correction

By J. R. POSTGATE

Chemical Research Laboratory, Teddington, Middlesex

SUMMARY: High cell densities reached in cultures of *Desulphovibrio desulphuricans* (Hildenborough strain), grown in a medium of glucose, peptone, yeast extract and salts were due to the presence of a non-sulphate-reducing anaerobic contaminant whose presence was detectable only in special conditions. The relevance of this to previous studies on the Hildenborough strain is discussed and certain quantitative data are corrected.

Studies on the nutrition of a strain of *Desulphovibrio desulphuricans* (Postgate, 1951*a*) appeared to indicate that a medium of glucose, salts, peptone and yeast extract permitted growth to about 750 μg. dry wt. cells/ml. Peptone and yeast extract acted synergistically; peptone was the more important constituent since, with high concentrations of peptone, yeast extract could be omitted, but the reverse was not true. Various pure vitamins, amino-acids, etc., were tested for their ability to replace the complex nitrogenous supplements, and a mixture of cysteine, serine, ornithine and isoleucine had some growth-promoting effect. Grossman & Postgate (1953) showed that the cysteine in this mixture could be replaced by Na_2S, indicating that the cysteine functioned through its reducing properties and not as a nutrient.

The present paper reports that the massive growth observed in the complex medium was partly due to the presence of an anaerobic contaminant in the stock strain used. The contaminant was only detectable in special conditions.

METHODS

Organism. A strain of *D. desulphuricans* called 'Hildenborough' was used throughout; its origin, maintenance and methods of subculture have been described (Postgate, 1951*a*). A stock was kept in a lyophilized condition, prepared by the procedure described in *Chemistry Research* (1951).

Media. Medium A consisted of glucose, peptone, yeast extract and sulphates (Postgate, 1951*a*); medium B consisted principally of sodium lactate, yeast extract and sulphates (Butlin, Adams & Thomas, 1949). When necessary, either medium was supplemented with one or more of the following: (*a*) agar (British Drug Houses Ltd.), 15 g./l. for solid media; (*b*) $FeSO_4.(NH_4)_2SO_4.6H_2O$ (0·5 g./l.) when it was desired to detect sulphate-reducing bacteria by blackening due to formation of FeS; (*c*) freshly sterilized and neutralized L-cysteine hydrochloride (5×10^{-3}M; British Drug Houses Ltd.) when it was desired that all viable organisms should grow (*Chemistry Research*, 1951; Grossman & Postgate, 1953). Reagents were of 'analytical reagent' grade except where stated.

Rough agar dilutions. A sealed Pasteur pipette was dipped into the culture being tested, and then dipped successively into seven soft-glass test tubes (15 × 1 cm.) each containing *c.* 4 ml. molten agar medium (40 ± 1°), supplemented with $FeSO_4.(NH_4)_2SO_4.6H_2O$. The medium was allowed to set and incubated in air at 30°. This procedure enabled populations to be checked rapidly for anaerobic contaminants, and gave well-separated black colonies of sulphate-reducing bacteria suitable for isolation in the third or fourth dilution. When the medium was supplemented with cysteine well-separated colonies appeared in the sixth or seventh dilution.

RESULTS

A stock of the Hildenborough strain maintained in medium A, which regularly reached high cell densities on subculture, appeared pure when plated out for aerobic and anaerobic contaminants on (1) medium A solidified with agar, (2) medium B solidified with agar, (3) blood agar, (4) glucose peptone agar. On testing by the agar dilution procedure in medium A solidified with agar and containing $FeSO_4.(NH_4)_2SO_4.6H_2O$ white colonies were sometimes observed among the black. The white colonies consisted of Gram-negative non-motile rods. Re-isolation of sulphate-reducing bacteria from the black colonies yielded a strain giving only limited growth in medium A; growth was restored to its former level when a culture derived from a white colony was added. Some properties of the purified and mixed strains are reported below.

The pure Hildenborough strain of *D. desulphuricans* consisted of typical motile vibrios with no tendency to pleomorphism. Growth in medium A reached a maximum of *c.* 350 μg. dry wt. cells/ml., but was nevertheless superior to growth in medium B (*c.* 100 μg./ml.). The cell density in medium A was increased by greater amounts of glucose and sulphate, a maximum of 500 μg./ml. being reached with glucose (80 μmol./ml.) + Na_2SO_4 (40 μmol./ml.). The cell density was not increased by culture in medium A in which the contaminant had been grown and filtered off. Growth of the purified strain in medium A without peptone and yeast extract was stimulated by a mixture of cysteine, serine, ornithine and *iso*leucine to the same extent as reported for the impure strain.

The hydrogenase activity of the pure strain was greater than the reported hydrogenase activity of the mixed strains; cell suspensions reduced sulphate in hydrogen with $-Q_{H_2}$ values of *c.* 700 mm.^{3} H_2/mg. dry wt./hr., compared with values of *c.* 200 for the mixed strain. Sulphite, thiosulphate and tetrathionate were also reduced; thiosulphate and tetrathionate were attacked more slowly than sulphate or sulphite as reported for the mixed strains (Postgate, 1951*b*).

The contaminant was an obligate anaerobic, Gram-negative, non-motile rod, often paired, which formed round or disk-shaped colonies with a perceptible yellowish tint in medium A agar + $FeSO_4(NH_4)_2SO_4.6H_2O$. It grew very feebly in liquid medium A and frequently died out on subculture; growth was improved by addition of the filtrate of a pure culture of Hildenborough grown in medium A. These observations suggest that there was some nutritional interdependence between the contaminant and the sulphate-reducing bacteria. The cell densities reached in medium A were too low to be measured accurately in a photo-electric turbidimeter; they were of the order of 30 μg. dry wt./ml. The organism did not grow in medium B.

Table 1. *Growth of purified and contaminated* Desulphovibrio desulphuricans *(Hildenborough) in various media*

Cultures were incubated until growth had ceased and the cell densities were determined photometrically.

Medium	Strain	Maximum cell density (μg. dry wt. cells/ml.)
A	Impure Hildenborough	780
A	Purified Hildenborough	270
A	Contaminant	*c.* 30
A	Purified Hildenborough + contaminant	735
B	Purified Hildenborough	120
B	Purified Hildenborough + contaminant	110
A*	Contaminant	90

* Medium A in which Hildenborough had been grown and filtered off.

The mixed strains (Postgate, 1951*a*) consisted wholly of Gram-negative organisms which appeared as a mixture of motile vibrios, non-motile rods and intermediate forms. The apparent proportion of rods to vibrios varied during growth, vibrios predominating in the stationary phase. The rods were often elongated and bent, and sometimes paired. This heteromorphic appearance was originally attributed to pleomorphism, since plate tests did not indicate any contaminants, but it is now clear that at least some of the rod-forms were contaminants. Cultures in medium A reached about 750 μg. dry wt. cells/ml., but cultures in medium B only reached cell densities similar to the pure strain in medium B. Moreover, cultures of the mixed strain in medium B appeared to consist wholly of vibrios; few, if any, rod forms could be seen. Nevertheless, even after many subcultures in medium B, the contaminant re-appeared immediately on subculture into medium A. The contaminant was also found by this procedure in a stock that had been maintained in Baars's (1930) medium for many years.

Other strains. Two other stock strains of *D. desulphuricans* out of six so far tested were found to be contaminated with an organism similar to that found with Hildenborough. Like Hildenborough, these strains had been checked by plating for aerobic and anaerobic contaminants at the time of their isolation or receipt. The El Agheila Z strain, which has been the subject of communications from this laboratory (Adams, Butlin, Hollands & Postgate, 1951; Postgate, 1952*b*, *c*) was pure.

DISCUSSION

The heteromorphic appearance of the Hildenborough strain was originally regarded as 'typical', and was attributed to the pleomorphism for which the genus *Desulphovibrio* is notable. In fact its 'pleomorphism' was due to the presence of a contaminant which was detectable culturally only in the special conditions described. The contaminant, like those described by Bunker (1939), stimulated growth of the sulphate-reducing bacteria in medium A, and its own growth in this medium was improved by sulphate-reducing bacteria. Moreover, though it did not grow alone in medium B, it nevertheless survived many subcultures in this medium provided sulphate-reducing bacteria were present. Clearly there was an effective syntrophism between the two bacteria; this made detection of the contaminant difficult.

In view of the presence of a contaminant in the Hildenborough strain of *D. desulphuricans*, certain quantitative data previously reported require correction. Fortunately the qualitative information given is unaffected.

The cell densities reached in medium A by *D. desulphuricans*, strain Hildenborough, are not as great as originally reported (Postgate, 1951*a*), but are nevertheless greater than in medium B. The growth-promoting effect of a mixture of cysteine, serine, ornithine and isoleucine is confirmed with the purified strain. This paper reported that, on one occasion, the Hildenborough strain suddenly lost its ability to give massive growth after more than ninety passages in medium A. This variation was not attributable to any change in the medium, cultural conditions or morphology of the strain; a new series from a stock in Baars's (1930) medium behaved 'normally'. In the light of the present observations it seems likely that the strain was inadvertently purified on this occasion, but that the consequent change to a more regular morphological appearance was overlooked. A return to the stock in Baars's medium gave the mixed population again.

A further paper (Postgate, 1951*b*) was concerned with the reduction of sulphur compounds by cell suspensions in hydrogen. Its general conclusions remain valid since the contaminant did not have hydrogenase activity, but, owing to dilution by contaminant, the specific activities of the sulphate-reducing bacteria in cell suspensions used were presumably greater than those quoted. The relative rates of attack of substrates by the pure strain were the same as with mixed population. Errors in growth experiments introduced by the presence of the contaminant cannot have been great or they would have been noticed in the appropriate controls.

Inhibition of growth and of hydrogen absorption by various sulphate analogues and other inhibitors was described, and the nature of the inhibitions studied (Postgate, 1949, 1952*a*). The conclusions reached in these papers remain valid, except that the specific cell activities reported in studies with suspensions were probably too low owing to dilution by the contaminant.

Postgate (1952*c*), Grossman & Postgate (1953), in the section entitled 'Fundamental studies of sulphate-reducing bacteria' in *Chemistry Research* 1952 and publications subsequent to these, referred to the purified strain. Earlier issues of *Chemistry Research* described work with the impure strain, though many of these studies used media in which the effect of the contaminant would be negligible.

The author is indebted to Dr J. Senez for introducing him to the procedure described as 'rough agar dilutions'. This paper is published by permission of the Director, Chemical Research Laboratory.

REFERENCES

ADAMS, M. E., BUTLIN, K. R., HOLLANDS, S. J. & POSTGATE, J. R. (1951). The rôle of hydrogenase in the autotrophy of *Desulphovibrio*. *Research, Lond.* **4**, 245.

BAARS, J. K. (1930). Over sulphaatreductie door bacterien, Dissertation, Delft.

BUNKER, H. J. (1939). Factors influencing the growth of *Vibrio desulphuricans*. *Int. Congr. Microbiol.* (New York, 1939), p. 64.

BUTLIN, K. R., ADAMS, M. E. & THOMAS, M. (1949). The isolation and cultivation of sulphate-reducing bacteria. *J. gen. Microbiol.* **3**, 46.

Chemistry Research 1952 and earlier issues. London: H.M. Stationery Office.

GROSSMAN, J. P. & POSTGATE, J. R. (1953). The cultivation of sulphate-reducing bacteria. *Nature, Lond.* **171**, 600.

POSTGATE, J. R. (1949). Inhibition of sulphate reduction by selenate. *Nature, Lond.* **164**, 670.

POSTGATE, J. R. (1951*a*). On the nutrition of *Desulphovibrio desulphuricans*. *J. gen. Microbiol.* **5**, 714.

POSTGATE, J. R. (1951*b*). The reduction of sulphur compounds by *Desulphovibrio desulphuricans*. *J. gen. Microbiol.* **5**, 725.

POSTGATE, J. R. (1952*a*). Competitive and non-competitive inhibitors of bacterial sulphate reduction. *J. gen. Microbiol.* **6**, 128.

POSTGATE, J. R. (1952*b*). Growth of sulphate-reducing bacteria in sulphate-free media. *Research, Lond.* **5**, 189.

POSTGATE, J. R. (1952*c*). The metabolism of pyruvate by sulphate reducers. *Int. Congr. Biochem.* (Paris, 1952), p. 92.

(*Received* 19 *May* 1953)

DEWEY, V. C. & KIDDER, G. W. (1953). *J. gen. Microbiol.* 9, 445–453.

Factors affecting the Requirement of *Tetrahymena pyriformis* (*geleii*) for Folic Acid

BY VIRGINIA C. DEWEY AND G. W. KIDDER
Biological Laboratory, Amherst College, Amherst, Massachusetts, U.S.A.

SUMMARY: In the use of an organism for assay of folic acid those compounds which affect the utilization of folic acid should be determined. With *Tetrahymena pyriformis* (*geleii*), it is shown that thymine (or thymidine), glycine, *p*-aminobenzoic acid, creatin and vitamin B_{12} diminish the requirement for folic acid. Pteroic acid, *p*-aminobenzoylglutamic acid, 5-methylcytosine, *p*-hydroxybenzoic acid, carbamylaspartic acid and ribonucleic acid have no effect on the requirement, while cholesterol and cortisone increase the requirement.

For some time the efforts of this laboratory have been turned toward the formulation of a technique by means of which the ciliate *Tetrahymena pyriformis* (*geleii*) (Corliss, 1952), could be utilized as a means of assay for folic acid and its conjugates. For this purpose the organism possesses a number of advantages: (*a*) it is able to utilize either free folic acid or its polyglutamic acid conjugates with equal facility (Kidder & Dewey, 1947); (*b*) it does not utilize either pteroic acid or the SLR factor (Kidder & Fuller, 1946) in place of folic acid; (*c*) folic acid cannot be replaced by a mixture of purines plus thymine (Kidder, Dewey, Parks & Heinrich, 1950). The organism shares with some bacteria the ability to grow in a medium of chemically-defined composition (Dewey, Parks & Kidder, 1950) and the ease with which measurements of growth may be made (Kidder & Dewey, 1948). However, in order to assay materials of plant or animal origin, it must first be determined whether or not such materials contain substances which will spare the requirement for folic acid or give non-specific stimulation. Since such materials undoubtedly contain many substances of unknown nature, one can only test a series of compounds which seem to be involved in folic acid metabolism. The presence of another member of the folic acid group, i.e. the citrovorum factor (Sauberlich & Baumann, 1948) must also be taken into account. It has been shown that this material has an activity for *T. pyriformis* somewhat inferior to that of folic acid (Kidder, Dewey & Parks, 1951*a*). It has recently been reported that the naturally occurring citrovorum factor has an activity double that of the synthetic material (Keresztesy & Silverman, 1951). This difference appears to be due to the fact that the synthetic material consists of a racemic mixture of which only one isomer has appreciable biological activity (Cosulich, Smith & Broquist, 1952). On this basis it would appear that natural citrovorum factor has an activity for *T. pyriformis* about equal to that of folic acid. It has been suggested that all of the folic acid, within certain cells at least, can be accounted for as citrovorum factor (Swendseid, Bethell & Ackermann, 1951), although later work shows that there is a fairly large amount of free folic acid in tissues and that both folic acid and citrovorum factor occur naturally as conjugates with glutamic acid (Wieland, Hutchings & Williams, 1952).

The present communication deals with the ability of many compounds to diminish the requirement of *T. pyriformis* for folic acid.

MATERIALS AND METHODS

The organism used was *Tetrahymena pyriformis* (*geleii*) W in pure bacteria-free culture. It was grown in medium A (Dewey *et al.* 1950) containing 10 mg. Tween 80/ml. in place of Tween 85. Folic acid (pteroylglutamic acid) was omitted from the medium and used as a variable component as has been described previously (Kidder, Dewey & Parks, 1951*b*). The range of concentrations tested was from 0·1 to 1·0 mμg./ml. To the basal medium additions were made as noted in the various experiments. All the experiments were done in triplicate and repeated a number of times.

The inoculum was prepared by transferring organisms grown in 2% proteose peptone into basal medium containing 0·5 mμg. folic acid/ml. This procedure is necessary to eliminate the 'carry-over' effect (Kidder, 1946). In certain experiments the age and size of the inoculum were varied to determine the effects of these factors on growth. A standard inoculum 7–9 days old at a dilution of 1/200 was used in most of the experiments. On a few occasions an inoculum grown in the complete absence (except for carry-over) of folic acid was used.

The medium was adjusted to pH 7·0 by means of a Beckman pH meter in order to protect the folic acid (the presence of Tween in the medium does not permit the accurate use of indicator dyes). Every possible effort was made to protect the media from light in order to minimize the destruction of folic acid. After inoculation the cultures were incubated in the dark at 25° in a sloped position for 96 hr. Readings of the turbidity produced by the growth of the organisms were made by means of a Lumetron photoelectric colorimeter with red filter no. 650.

RESULTS

In view of the many factors which affect the response of *T. pyriformis* to folic acid and produce variability in the results, typical experiments have been chosen to illustrate the effects of materials added to the medium. The same experiment done at different times may give somewhat different results quantitatively, although they have been repeated frequently enough to be certain of the qualitative responses.

Data obtained by varying the age of the inoculum indicate that the folic acid requirement decreases to a minimum when the inoculum is 7–9 days old, being greater with either older or younger inocula. When inocula are less than 7 days old this may be due to the fact that the inoculum is smaller. Inocula of 1/500 or 1/1000 also exhibit a larger folic acid requirement. This is not attributable to carry-over of folic acid as such in the medium, since essentially the same results are obtained using first transplant inocula grown in the absence of added folic acid (Kidder, 1946). Occasionally the older inocula failed to grow when transplanted into media containing even supraoptimal amounts of folic acid (1 mμg./ml.), although organisms from the same cultures grew rapidly when transplanted into proteose peptone. Folic acid has a large effect on the rate of growth of a culture of *T. pyriformis*. Suboptimal concentrations of folic acid permit only slow growth, but the maximum yield of organisms eventually reaches nearly that attained in higher concentrations (Fig. 1). Thus it is apparent that the time at which the amount of growth is determined will have an influence on the apparent magnitude of the requirement for folic acid.

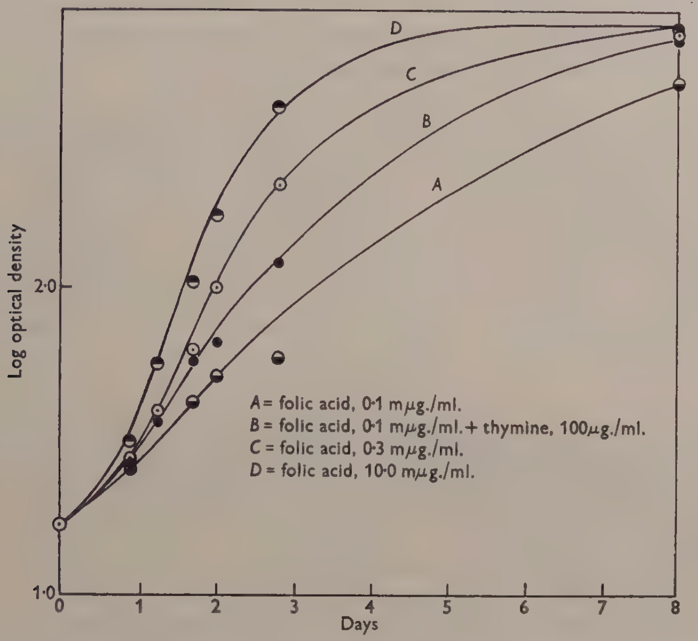


Fig. 1. **Influence of folic acid and thymine on growth rate of *T. pyriformis*.**

It was shown some years ago that folic acid could be replaced in the nutrition of *Lactobacillus casei* and *Streptococcus faecalis* by a mixture of thymine and purines (Stokstad, 1941; Snell & Mitchell, 1941). It has already been demonstrated that *T. pyriformis* requires folic acid in addition to purines and thymine (Kidder *et al.* 1950), although thymine stimulates growth. Table 1 shows the degree of stimulation given by thymine in the presence of low concentrations of folic acid. It can be seen that the addition of thymine to the medium greatly increases the blank reading. This blank is quite variable and in some cases higher than half-maximal growth. Nevertheless, media which produce such growth are incapable of supporting a second transplant; this must be attributed to the sparing of folic acid carried over in the inoculum. Thymidine is even more effective than thymine in sparing folic acid and for *Leuconostoc citrovorum* it can replace folic acid entirely (Sauberlich, 1949). In contrast, 5-methylcytosine has no influence on the folic acid requirement of *T. pyriformis*. This compound, like cytosine, is apparently not deaminated. It seems clear that one of the functions of folic acid in the nutrition of *T. pyriformis* is in the synthesis of thymine or of thymidine. One of the suggested intermediates in the synthesis of pyrimidines by micro-organisms is ureidosuccinic acid (carbamylaspartic acid; Wright, Valentik, Spicer, Huff & Skeggs, 1950). For this reason carbamylaspartic acid was tested for its ability to replace either uracil or thymine in the growth of *T. pyriformis*. It was found to be entirely without activity. This was not entirely unexpected in view of the inactivity of orotic acid, of which ureidosuccinic acid is a precursor with *Lactobacillus bulgaricus*.

Table 1. *The influence of thymine on the folic acid requirement of* Tetrahymena pyriformis

Addition (μg./ml.)	Folic acid required for half-maximal growth (mμg./ml.)	Blank reading, optical density
None	0·320	0·056
Thymine		
10	0·190	0·102
20	0·180	0·117
30	0·175	0·128
40	0·170	0·123
Thymidine		
5	0·075	0·194

While *p*-aminobenzoic acid (*p*-AB) forms a part of the folic acid molecule, there have been suggestions (Lampen & Jones, 1947; Sarrett 1947) that *p*-AB has a function of its own. The effect of adding *p*-AB to media low in folic acid was tested, and it was found to increase growth slightly over that of controls (Table 2). This effect was additive to that obtained by the addition of thymine and glycine. While the addition of the nucleotides derived from nucleic acid had no effect alone, they improved growth when added in the presence of *p*-AB. The significance of these results is not yet clear. They suggest that if *p*-AB is required, it is obtained by the breakdown of the folic acid molecule or that

Table 2. *The effect of nucleic acid derivatives on the folic acid requirement of* Tetrahymena pyriformis

			Nucleic acid derivatives*			
Additions (mμg./ml.)	None		None		Glycine and thymine†	
	a	*b*	*a*	*b*	*a*	*b*
None	0·410	0·056	0·440	0·062	0·315	0·123
p-Aminobenzoic acid						
0·1	0·360	0·057	0·385	0·058	0·270	0·128
0·5	0·320	0·053	0·296	0·065	0·250	0·114
1·0	0·325	0·058	0·296	0·058	0·200	0·127
10·0	0·330	0·061	0·290	0·064	0·175	0·128
100·0	0·350	0·059	0·290	0·068	0·160	0·138

* Nucleic acid derivatives used in the basal medium at tenfold concentration.

† Thymine, 40 μg./ml.; glycine, 200 μg./ml.

a = mμg. folic acid/ml. required for half maximal growth.

b = Optical density readings of blank cultures.

it is present as a trace contaminant of the folic acid used. It would appear that its function is not concerned with the metabolism of glycine or of the purines or pyrimidines. It is interesting that *p*-hydroxybenzoic acid is entirely inert under these conditions.

Pteroic acid (*p*-aminobenzoylpteridine) has no sparing action on folic acid, nor has rhizopterin (SLR factor, formylpteroic acid), although both are capable of replacing folic acid in the growth of *Streptococcus faecalis* R (Stokes, Keresztesy & Foster, 1944). Pteroic acid or SLR factor isolated from natural sources were, however, capable of replacing folic acid when used at sufficiently high levels, but this is undoubtedly due to contamination of such preparations with folic acid. On the other hand, *p*-aminobenzoylglutamic acid does not influence the folic acid requirement of *T. pyriformis.*

The work of Stekol & Weiss (1950) and of Stekol, Weiss & Weiss (1951) showed that the utilization of glycine as a source of labile methyl groups in mammalian nutrition is dependent on the presence of folic acid. That glycine also spares folic acid in the nutrition of *T. pyriformis* is shown in Table 3. This may be related to the fact that serine synthesis from glycine fails except in the presence of extremely high levels of folic acid (Kidder & Dewey, 1953). It would appear that conversion of serine to glycine is also under the control of folic acid. It may also be seen (Table 2) that the action of thymine, glycine and *p*-AB are additive.

Table 3. *The influence of glycine on the folic acid requirement of* Tetrahymena pyriformis

			Thymine		*p*-AB			
	None				None		Thymine	
Additions			None	*p*-AB				
(μg./ml.)	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
None	0·265	0·079	0·100	0·193	0·205	0·114	0·003	0·347
Glycine								
10	0·195	0·083	0·090	0·217	—	—	—	—
25	0·195	0·094	0·060	0·229	—	—	—	—
50	0·180	0·087	0·045	0·242	—	—	—	—
100	0·175	0·087	0·042	0·253	0·115	0·130	*	0·377
200	0·170	0·100	0·030	0·262	—	—	—	—

* Could not be determined because blank reading was greater than half-maximal.
a = Amount of folic acid required for half-maximal growth in mμg./ml.
b = Optical density readings of blank cultures.
Thymine concentration, 40 μg./ml., *p*-AB, 1 μg./ml.

It has been reported that the sterols, dehydro*iso*androsterone and cortisone (Gaines & Totter, 1950; Gaines & Broquist, 1951) can replace folic acid in the nutrition of *Lactobacillus casei*. The effect of the addition of either cortisone or cholesterol on the response of *T. pyriformis* to folic acid is shown in Fig. 2. While cortisone at 100 μg./ml. is inhibitory, lower concentrations had no effect on growth. Cholesterol was somewhat less inhibitory, and in the presence of adequate folic acid was even slightly stimulatory. The addition of glycine and thymine to the medium did not overcome the inhibitory effect of cortisone, although it did that of cholesterol. Dehydro*iso*androsterone was not tested.

A discussion of the interrelationships involving the steroid hormones, folic acid and ascorbic acid may be found in Lloyd & Sinclair (1953).

In Table 4 are listed a number of other compounds which might possibly affect or be affected by folic acid. Betaine (Dubnoff, 1949) and formate (Sakami, 1948) are known to be sources of labile methyl groups in the mammal, but it is apparent that they have little, if any, effect on the folic acid requirement of *T. pyriformis*. It has been noted that excess creatin is excreted by the monkey in folic acid deficiency (Dinning & Day, 1949). Since creatin is a methylated compound it might spare folic acid in transmethylation reactions. This may, indeed, be the explanation of the slight sparing effect exhibited by creatin. Vitamin B_{12} is believed to function in the synthesis of thymidine (Wright, Skeggs & Huff, 1948) or other deoxyribosides (Skeggs, Spizizen & Wright, 1950), and it is, therefore, to be expected that it might function also in the sparing of folic acid. This action was noted (Table 4).

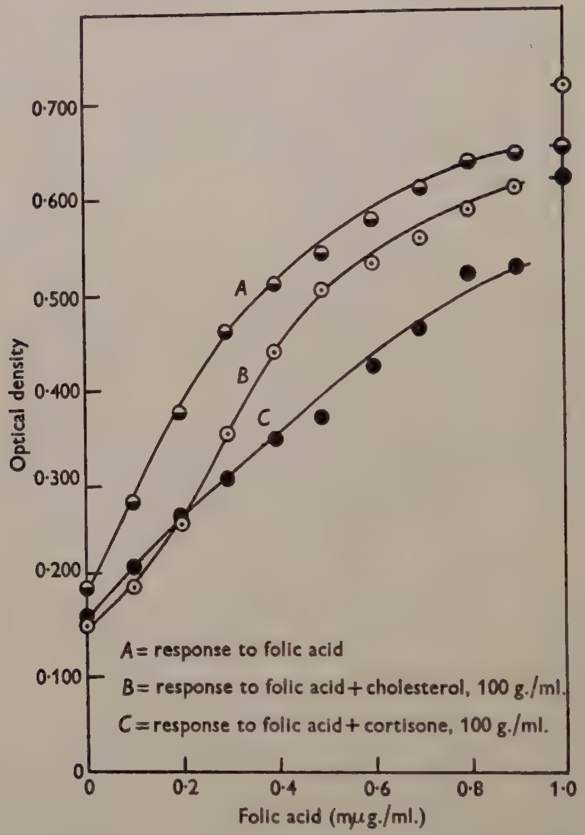


Fig. 2. Influence of sterols on response of *T. pyriformis* to folic acid.

The ability of preparations of ribonucleic acid (RNA) to spare folic acid and at high levels even to replace it, can be shown to be due to the presence of folic acid as a contaminant. Purification of commercial samples of RNA by repeated reprecipitation with ethanol or with acid gave material which had no sparing effect on folic acid. Similar treatment of commercial deoxyribonucleic acid (DNA) was not successful in removing the activity, but purer samples isolated in the laboratory gave results which could be explained solely on the basis of the thymidine content of DNA. It seems probable that the reported ability of *T. pyriformis* to grow in the absence of certain vitamins (Elliott, 1950; Seaman, 1953) can be attributed to the use of contaminated RNA in the medium.

Table 4. *Comparison of effects of various compounds on the folic acid requirement of* Tetrahymena pyriformis

Addition (μg./ml.)	Folic acid required for half-maximal growth (mμg./ml.)	Blank reading, optical density	Maximum reading, optical density
None	0·214	0·086	0·626
Betaine, 40	0·230	0·095	0·600
Cholesterol, 10	0·245	0·085	0·585
Haemin, 10	0·238	0·148	0·620
Formate, 100	0·190	0·102	0·581
Creatin, 40	0·155	0·110	0·591
Glycine, 100	0·140	0·100	0·623
Thymine, 40	0·065	0·238	0·629
Glycine, 100; thymine, 40	0·040	0·251	0·585
None	0·143	0·118	0·614
Vitamin B_{12}, 0·001	0·086	0·154	0·617

In order to perform assays for folic acid, its conjugates or citrovorum factor, by the use of *T. pyriformis*, it would be necessary to include in the medium all of the substances mentioned above which have shown a sparing effect, and to prepare an inoculum which would give a low blank reading without decreased vitality. On the other hand, since *T. pyriformis* exhibits a typical animal pattern in its folic acid nutrition, as well as in other respects, assays in the basal medium would give results indicative of the total folic acid activity of the material assayed. One major difference between the ciliate and the mammal lies in the inability of *T. pyriformis* to transmethylate homocysteine (Genghof, 1949), a reaction which is influenced by folic acid. Another major difference is the inability of the mammal to utilize exogenous thymine (Plentl & Schoenheimer, 1944). This discrepancy can be readily overcome by the inclusion of thymine in the assay medium. The main advantages of the use of *T. pyriformis* rather than the various lactobacilli for folic acid assays lies in the ability of the ciliate to utilize conjugates of folic acid without previous digestion. Bacterial assays would still be necessary in order to differentiate between folic acid and the citrovorum factor.

The only naturally occurring compound which will replace folic acid in the nutrition of *T. pyriformis* is the citrovorum factor (5-formyl-5:6:7:8-tetrahydropteroylglutamic acid) or the conjugates of these compounds. The earlier report (Kidder *et al.* 1951*a*) that aminopterin could replace folic acid for growth was due to gross contamination of the sample of aminopterin used with folic acid (Heinrich, Dewey & Kidder, 1953). It seems probable that other folic acid analogues which promote growth of *T. pyriformis* are also contaminated.

This work was aided by grants from the Research Corporation and from the American Cancer Society recommended by the Committee on Growth. We gratefully acknowledge gifts of folic acid and pteroic acid from the Lederle Laboratories, Pearl River, New York; carbamylaspartic acid from Dr D. Sprinson, Columbia University, New York City, New York; thymidine from Dr E. E. Snell, University of Texas, Austin, Texas. Dr R. E. Parks, Jr., participated in the early phases of this work.

REFERENCES

Corliss, J. O. (1952). Comparative studies on holotrichous ciliates in the Colpidium-Glaucoma-Leucophrys-Tetrahymena group. I. General considerations and history of strains in pure culture. *Trans. Amer. micr. Soc.* **71**, 159.

Cosulich, D. B., Smith, J. M. Jr. & Broquist, H. P. (1952). Diastereoisomerism of leucovorin. *J. Amer. chem. Soc.* **74**, 4215.

Dewey, V. C., Parks, R. E. Jr. & Kidder, G. W. (1950). Growth responses of *Tetrahymena geleii* to changes in the basal medium. *Arch. Biochem.* **29**, 281.

Dinning, J. S. & Day, P. L. (1949). Creatinuria during recovery from Aminopterin induced folic acid deficiency in the monkey. *J. biol. Chem.* **181**, 897.

Dubnoff, J. (1949). The role of choline oxidase in labilizing choline methyl. *Arch. Biochem.* **24**, 251.

Elliott, A. M. (1950). The growth-factor requirements of *Tetrahymena geleii* E. *Physiol. Zoöl.* **23**, 85.

Gaines, D. S. & Broquist, H. (1951). Microbial activity of cortisone acetate. *Fed. Proc.* **10**, 186.

Gaines, D. S. & Totter, J. R. (1950). Pteroylglutamic acid-like effect of dehydroisoandrosterone on growth of certain microorganisms. *Proc. Soc. exp. Biol., N.Y.* **74**, 558.

Genghof, D. S. (1949). The sulfur amino acid requirements of *Tetrahymena geleii. Arch. Biochem.* **23**, 85.

Heinrich, M. R., Dewey, V. C. & Kidder, G. W. (1953). Ion exchange in chromatography of pteroylglutamic acid and Aminopterin. *J. Amer. chem. Soc.* (In the Press.)

Keresztesy, J. C. & Silverman, M. (1951). Crystalline citrovorum factor from liver. *J. Amer. chem. Soc.* **73**, 5510.

Kidder, G. W. (1946). Studies on the biochemistry of *Tetrahymena*. VI. Folic acid as a growth factor for *T. geleii* W. *Arch. Biochem.* **9**, 51.

Kidder, G. W. & Dewey, V. C. (1947). Studies on the biochemistry of *Tetrahymena*. IX. Folic acid components and conjugates. *Proc. nat. Acad. Sci., Wash.* **33**, 95.

Kidder, G. W. & Dewey, V. C. (1948). Dietary factors in the utilization of homocystine. *Proc. nat. Acad. Sci., Wash.* **34**, 81.

Kidder, G. W. & Dewey, V. C. (1953). Influence of thioctic acid and folic acid on amino acid synthesis in Tetrahymena. *Fed. Proc.* **12**, 230.

Kidder, G. W., Dewey, V. C. & Parks, R. E. Jr. (1951*a*). Growth promotion in *Tetrahymena* by folic acid analogs. *Proc. Soc. exp. Biol., N.Y.* **78**, 88.

Kidder, G. W., Dewey, V. C. & Parks, R. E. Jr. (1951*b*). Studies on the inorganic requirements of *Tetrahymena. Physiol. Zoöl.* **24**, 69.

Kidder, G. W., Dewey, V. C., Parks, R. E. Jr. & Heinrich, M. R. (1950). Further studies on the purine and pyrimidine metabolism of *Tetrahymena. Proc. nat. Acad. Sci., Wash.* **36**, 431.

Kidder, G. W. & Fuller, R. C. III (1946). The growth response of *Tetrahymena geleii* W to folic acid and to the *Streptococcus lactis* R factor. *Science*, **104**, 160.

Lampen, J. O. & Jones, M. J. (1947). The growth promoting and antisulfonamide activity of *p*-aminobenzoic acid, pteroylglutamic acid and related compounds for *Lactobacillus arabinosus* and *Streptobacterium plantarum*. *J. biol. Chem.* **170**, 133.

Lloyd, B. B. & Sinclair, H. M. (1953). In *Biochemistry and Physiology of Nutrition*, eds Bourne, G. and Kidder, G. W. New York: Academic Press.

Plentl, A. A. & Schoenheimer, R. (1944). Studies in the metabolism of purines and pyrimidines by means of isotopic nitrogen. *J. biol. Chem.* **153**, 203.

Sakami, W. (1948). The conversion of formate and glycine to serine and glycogen in the intact rat. *J. biol. Chem.* **176**, 995.

Sauberlich, H. E. (1949). The relationship of folic acid, vitamin B_{12} and thymidine in the nutrition of *Leuconostoc citrovorum* 8081. *Arch. Biochem.* **24**, 224.

Sauberlich, H. E. & Baumann, C. A. (1948). A factor required for the growth of *Leuconostoc citrovorum*. *J. biol. Chem.* **176**, 165.

Sarett, H. P. (1947). Interrelationship between *p*-aminobenzoic acid and pteroylglutamic acid as growth factors for lactobacilli. *J. biol. Chem.* **171**, 265.

Seaman, G. R. (1953). Synthesis of B vitamins by *Tetrahymena geleii* S. *Physiol. Zoöl.* **26**, 22.

Skeggs, H. R., Spizizen, J. & Wright, L. D. (1950). Competitive antagonism of ribonucleic and desoxyribonucleic acids in the nutrition of *Lactobacillus bifidus*. *J. Amer. chem. Soc.* **72**, 811.

Snell, E. E. & Mitchell, H. K. (1941). Purine and pyramidine bases as growth substances for lactic acid bacteria. *Proc. nat. Acad. Sci., Wash.* **27**, 1.

Stekol, J. A. & Weiss, K. (1950). Vitamin B_{12} and growth of rats on diets free of methionine and choline. *J. biol. Chem.* **186**, 343.

Stekol, J. A., Weiss, K. & Weiss, J. (1951). Role of folacine and vitamin B_{12} in synthesis and utilization of choline by the rat as studied with C^{14}-glycine, formate and methionine. *Fed. Proc.* **10**, 343.

Stokes, J. L., Keresztesy, J. C. & Foster, J. W. (1944). Relation of the *Streptococcus lactis* R factor to 'folic acid'. *Science*, **100**, 522.

Stokstad, E. L. R. (1941). Isolation of a nucleotide essential for the growth of *Lactobacillus casei*. *J. biol. Chem.* **139**, 475.

Swendseid, M. E., Bethell, F. H. & Ackermann, W. W. (1951). The intracellular distribution of vitamin B_{12} and folinic acid in mouse liver. *J. biol. Chem.* **190**, 791.

Wieland, O. P., Hutchings, B. L. & Williams, J. H. (1952). Studies on the natural occurrence of folic acid and the citrovorum factor. *Arch. Biochem. Biophys.* **40**, 205.

Wright, L. D., Skeggs, H. R. & Huff, J. W. (1948). The ability of thymidine to replace vitamin B_{12} as a growth factor for certain lactobacilli. *J. biol. Chem.* **175**, 475.

Wright, L. D., Valentik, K. A., Spicer, D. S., Huff, J. W. & Skeggs, H. R. (1950). Orotic acid and related compounds in the nutrition of *Lactobacillus bulgaricus* 09. *Proc. Soc. exp. Biol., N.Y.* **75**, 293.

(*Received* 26 *May* 1953)

FEW, A. V. & SCHULMAN, J. H. (1953). *J. gen. Microbiol.* 9, 454–466.

The Absorption of Polymyxin E by Bacteria and Bacterial Cell Walls and its Bactericidal Action

BY A. V. FEW AND J. H. SCHULMAN

Department of Colloid Science, University of Cambridge

SUMMARY: Isotherms of the absorption of polymyxin E by *Bacillus subtilis*, *Pseudomonas denitrificans* and *Streptococcus faecalis* after 20 min. contact with the antibiotic were determined. Tested on seven different bacteria, the maximum amounts of polymyxin E absorbed showed that the polymyxin-sensitive bacteria were capable of absorbing much greater quantities of the antibiotic than resistant organisms. When the bacteria were suspended in buffer solutions, the ultraviolet spectra of their supernatant fluids showed a maximum absorption at 260 mμ.; purines and pyrimidines contribute to this maximum. Addition of polymyxin E to suspensions of sensitive bacteria caused an immediate increase in the height of this maximum. Similar treatment of resistant organisms caused very little immediate increase in the amount of 260 mμ. absorbing material released. On prolonged incubation of untreated and polymyxin E treated cells there was a steady increase in the amount of 260 mμ. absorbing cell solutes released into the suspension media. Within the pH range 4·4–7·6 no optimum pH value was found for the polymyxin E absorption process or for the cellular release mechanism. The absorption isotherms of polymyxin E obtained with cell-wall preparations derived from sensitive and resistant bacteria showed that cell walls derived from sensitive organisms bound more polymyxin E than cell-wall preparations from resistant organisms. On treatment of sensitive bacteria with concentrations of polymyxin E less than those required for 99 % killing in 20 min. at 25°, there was a linear relationship between the amounts of 260 mμ. absorbing material released and the percentage of cells killed. Complete saturation of sensitive organisms with the antibiotic was not necessary to kill the cells. The amounts of polymyxin E required for 99·5 % killing corresponded closely to those sufficient for the formation of a closely packed monolayer within the bacterial cell wall. It is suggested that, at these bactericidal concentrations, polymyxin E combines with and thereby disorganizes the structures responsible for the maintenance of osmotic equilibrium within the cell wall.

The surface-active cationic polypeptide antibiotics of the polymyxin group form stable unimolecular films at the air/water interface when spread upon concentrated ammonium sulphate solution (Few & Schulman, 1953*a*). This observation provides a simple technique for the assay of these antibiotics and makes possible a detailed investigation of the absorption of these agents by susceptible and resistant bacteria and bacterial components.

The five members of this group of antibiotics (polymyxin A, B, C, D and E) were shown by Brownlee, Bushby & Short (1952) to possess similar antibacterial spectra and potency, whilst considerable evidence has accumulated showing that they are rapidly bactericidal and not bacteriostatic (Brownlee & Bushby, 1948; White, Alverson, Baker & Jackson, 1949; Bliss, Chandler & Schoenbach, 1949). White *et al.* (1949) demonstrated the bactericidal and bacteriolytic action of polymyxins A and D, and also found that electron micrographs of susceptible bacteria exposed to the antibiotics were much less electron dense than those of untreated cells. In microbiological assay procedures, the dependence of the minimum lethal concentration of polymyxin upon the size of the inoculum suggests that bacterial absorption of the antibiotic is high. In particular, the treatment of polymyxin with washed resting suspensions of a susceptible organism resulted in a loss of antibacterial activity, whereas similar treatment of solutions with a resistant organism caused no loss of activity (Bliss *et al.* 1949). This was interpreted as the result of absorption of the antibiotic upon the bacterial bodies of the susceptible organisms.

Phosphatides of the lecithin type interfere with the antibacterial action of polymyxin (Bliss *et al.* 1949), and in this respect polymyxin exhibits properties similar to those of the cationic detergents (Baker, Harrison & Miller, 1941). Rybak (1950) showed that insoluble complex formation takes place between polymyxin and serum albumin; a similar action also occurs with yeast ribonucleic acid (Latterade & Macheboeuf, 1950).

Although there is a similarity in the charge of the cationic detergents, the basic polypeptide tyrocidin and polymyxin, the cationic detergents and tyrocidin are more active against the Gram-positive bacteria, whilst polymyxin is, with some exceptions, only active towards the Gram-negative bacteria. By chemical analysis of a range of bacterial cell walls, Salton (1953) has shown that the cell-wall preparations from the Gram-positive bacteria contain very much less lipid than those derived from the Gram-negative organisms. From studies on the absorption of cetyltrimethylammonium bromide (CTAB) by bacteria and its action in releasing soluble cellular components, Salton (1951) supported the view expressed by Baker *et al.* (1941) that the primary action of surface active agents is that of irreversible damage to the cellular membrane, resulting in the release of cell constituents and dilution of these into the surrounding fluid. Treatment of susceptible bateria with tyrocidin also causes the release of cellular constituents (Gale & Taylor, 1947).

The present communication describes experiments on the absorption of polymyxin E by sensitive and resistant bacteria and bacterial cell walls, and on its action in releasing cellular constituents possessing a maximum absorption in their ultraviolet spectra at 260 mμ.

EXPERIMENTAL

Polymyxin E. A crystalline sample of polymyxin E sulphate, kindly given by Dr J. W. Trevan, F.R.S., Wellcome Research Laboratories, Langley Court, Beckenham, Kent, was used.

Organisms. Strains of *Pseudomonas denitrificans, Bacillus subtilis, Bacterium coli, Micrococcus lysodeikticus, Staphylococcus aureus, Streptococcus faecalis* and *Proteus vulgaris*, kindly given by Dr E. F. Gale, were used in this investigation. Determination of the sensitivities of these organisms to polymyxin E, by streaking 24 hr. broth cultures upon nutrient agar containing increasing quantities of the antibiotic, showed that the first four organisms were inhibited by 5 μg./ml., whilst the others were not inhibited by 100 μg./ml.

Preparation of bacterial suspensions. The organisms were grown in surface culture on a beef tryptic digest agar medium contained in 20 oz. bottles. The bottles were inoculated with *c.* 3 ml. of a 24 hr. broth culture, and the organisms were harvested after 16 hr. growth at 37°. The bacteria were washed 4 times by centrifugation in 0·01 M-phosphate buffer (pH 6·3), unless otherwise stated, and finally re-suspended in this medium at a concentration equivalent to 3 mg. dry wt. bacteria/ml. The dry weights of bacteria and of bacterial cell walls were determined directly by drying 1 ml. samples to constant weight in an air-oven at 105°.

Bacterial cell walls. Bacterial cell walls were prepared by disintegration of cells by shaking with no. 12 glass 'ballotini' beads (English Glass Co. Ltd., Empire Road, Leicester) in a Mickle disintegrator. By shaking 9 ml. of a 10 mg. dry wt./ml. bacterial suspension in 0·01 M-phosphate buffer (pH 6·3) with an equal volume of beads more than 95% of the cells were disrupted after 5 min. The progress of disruption could be followed qualitatively by the examination of Gram-stained smears. The glass beads were removed on a sintered glass filter and the suspensions were then centrifuged at low speed to remove debris and intact bacteria. Centrifugation of the supernatant at 3000 *g* caused the deposition of the cell-wall fraction, which was washed thoroughly by centrifugation with the phosphate buffer. The cell walls were finally resuspended in this medium and the dry weight determined. With the assistance of Mr H. Pearson of the Cavendish Laboratory, these preparations were examined in the electron microscope; this showed that less than 1% of intact cells were present. Provided the bacteria were disrupted in 0·01 M-phosphate buffer and subsequently thoroughly washed with this medium no electron-dense cytoplasmic material was found adhering to the cell walls.

Langmuir-Adam surface-balance method for the assay of polymyxin E solutions. Polymyxin E forms stable unimolecular films at the air/water interface when 70% (w/w) ammonium sulphate solution is used as the aqueous phase in the trough (Few & Schulman, 1953*a*). At a surface pressure of 16 dynes/cm., where compressibility of the film is at a minimum, 1 mg. polymyxin E occupies an area of 0·80 sq.m. To determine the concentration of polymyxin E in aqueous solution, a sample is spread on the $(NH_4)_2SO_4$ trough solution, and the area occupied by the unimolecular film at a surface pressure of 16 dynes/cm. is noted. By comparison of this area with that occupied by 1 mg. of antibiotic the concentration of the polymyxin solution can be calculated. This method is rapid, requires less than 0·5 ml. of solution, and can estimate the polymyxin at a concentration of 5 μg./ml. to ±10% and at 100 μg./ml. to ±1% (Few & Schulman, 1953*b*).

Examination of soluble cellular constituents in the supernatant fluid of bacterial suspensions. The ultraviolet spectra of the supernatant fluids obtained from buffered suspensions of untreated and polymyxin E treated cells were investigated by means of a Unicam quartz spectrophotometer. Where necessary, corrections were applied for the ultraviolet absorption of polymyxin E.

Absorption of polymyxin E by bacteria and bacterial cell walls. Unless otherwise stated, bacteria and bacterial cell walls were re-suspended to *c.* 1 mg. dry wt./ml. in 0·01 M-phosphate buffer (pH 6·3) alone and in buffered solutions of polymyxin E. Samples (3 ml.) were incubated in a water-bath at 25°. The organisms, or cell walls, were then removed by centrifugation and the supernatant fluids cleared by recentrifuging. Samples of these solutions were then assayed for unabsorbed polymyxin E by the surface balance method, and for the presence of released cellular constituents by means of the Unicam spectrophotometer. Control experiments in the absence of polymyxin E showed that in general the amount of cellular material released during the incubation period of 20 min. and which formed an insoluble monolayer on the $(NH_4)_2SO_4$ trough solution was small, but where necessary a correction for this was applied in the calculation of the amount of polymyxin E absorbed. Furthermore, with certain organisms almost complete bacterial absorption of the polymyxin E occurred, and the trough areas were no greater than those of the controls. This indicated that the ultraviolet absorbing cellular material released by susceptible bacteria in the presence of the antibiotic did not form monolayers upon the trough solution and hence did not interfere with the assay method.

Plate count estimation of surviving cells. Samples (1 ml.) of untreated and polymyxin E treated bacterial suspensions were withdrawn after 20 min. incubation at 25° and added to 9 ml. sterile broth. Further dilutions were then made in broth diluted with 3 vol. of sterile distilled water, and plated out in duplicate on agar medium. The colonies were counted after 24 hr. incubation at 37°.

Surface tensions of polymyxin E solutions. The surface tensions of buffered solutions of polymyxin E were determined at the air/water and *n*-hexane/water interfaces using the micrometer syringe drop volume method. The corrections discussed by Harkins & Brown (1919) were applied in the calculation of the interfacial tensions. Measurements were carried out at 20°.

RESULTS

Absorption of polymyxin E by bacteria

In Fig. 1 the absorption of polymyxin E by *Ps. denitrificans*, *B. subtilis* and *Strep. faecalis* is expressed as a function of the polymyxin concentration in the supernatant. The results show that marked differences existed, both in the shape of the absorption isotherms and in the amounts absorbed by the polymyxin-sensitive organisms, *Ps. denitrificans* and *B. subtilis*, when compared with the isotherm obtained with the polymyxin-resistant *Strep. faecalis*. The initial absorption process was complete at a supernatant concentration of *c.* 50 μg. polymyxin E/ml.; thereafter further absorption was slight. Table 1 shows the maximum amount of polymyxin E absorbed in 20 min. at 25° for a number of sensitive and resistant bacteria. In general the sensitive bacteria absorbed approximately four times as much polymyxin as the resistant organisms. Of four strains of *Bact. coli* investigated, three were agglutinated in the presence of the antibiotic, and complete saturation of the organisms with polymyxin E was not obtained even at concentrations of 800 μg. polymyxin/ml. of supernatant.

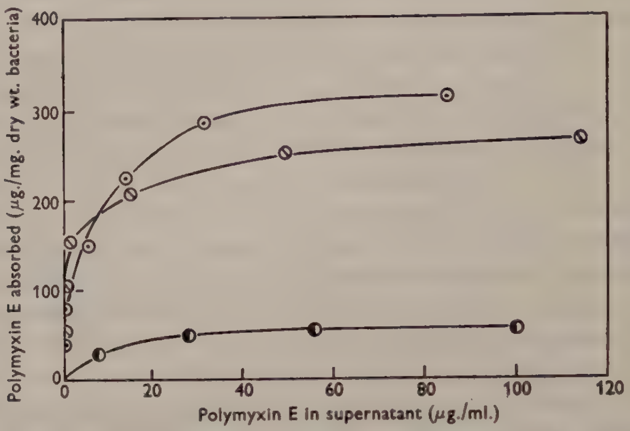


Fig. 1. Absorption of polymyxin E by bacteria. Organisms were incubated at 25° for 20 min. in 0·01 M-phosphate buffer pH 6·3 containing polymyxin E. – ◐ –, *Strep. faecalis*; – ⊘ –, *B. subtilis*; – ⊙ –, *Ps. denitrificans*.

Table 1. *Maximum amounts of polymyxin E absorbed by bacteria in* 20 *min. at* 25°, *its relation to their Gram reaction and polymyxin sensitivity*

Suspensions in 0·01 M-phosphate buffer pH 6·3.

Organism	Gram reaction	Polymyxin sensitivity	Maximum amount of polymyxin E absorbed (μg./mg. dry wt. bacteria)
Ps. denitrificans	–	Sensitive	350
M. lysodeikticus	+	Sensitive	375
Bact. coli	–	Sensitive	>220
B. subtilis	+	Sensitive	310
Strep. faecalis	+	Resistant	71
Staph. aureus	+	Resistant	84
Pr. vulgaris	–	Resistant	82

Release of ultraviolet absorbing material by bacteria

Examination of the ultraviolet spectra of the supernatant fluids from buffered suspensions of *Ps. denitrificans* alone, and in the presence of polymyxin E, revealed a maximum in their absorption spectra at 260 mμ. The presence of free purines and pyrimidines in the supernatant fluids contribute to this maximum (Dehéré, 1906; Salton, 1951). Fig. 2 shows the ultraviolet spectra of these fluids; a marked increase in the 260 mμ. absorption maximum occurred with the cells treated with polymyxin E. Similar results were obtained with other polymyxin-sensitive bacteria, whereas with suspensions of resistant organisms the addition of polymyxin caused only a very small increase in this absorption maximum. In Fig. 3 the release of 260 mμ. absorbing cellular material is plotted as a function of the amount of polymyxin E added to

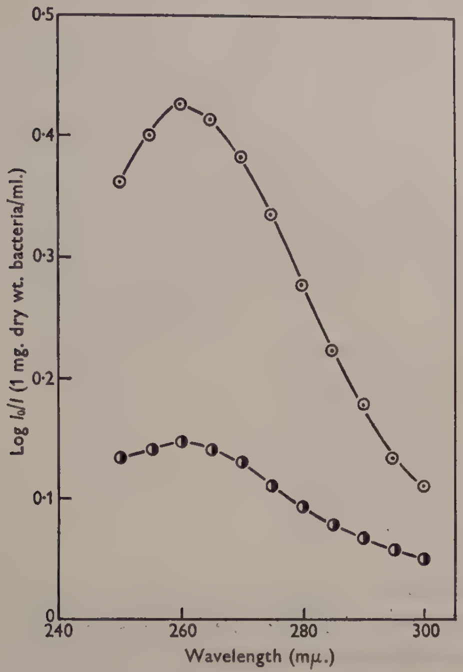


Fig. 2. Ultraviolet spectra of supernatant fluids from *Ps. denitrificans* suspensions (1 mg. dry wt. bacteria/ml.) incubated at 25° for 20 min. in 0·01 M-phosphate buffer (pH 6·3). – ◑ –, untreated cells; – ⊙ –, cells treated with 30 μg. polymyxin E.

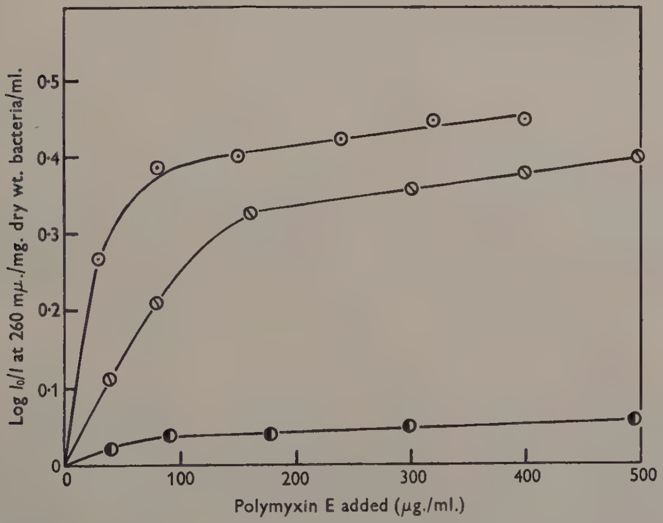


Fig. 3. Effect of the addition of polymyxin E on the release of 260 mμ. absorbing material by bacterial suspensions. Organisms were incubated at 25° for 20 min. in 0·01 M-phosphate buffer pH 6·3 containing polymyxin E. – ◐ –, *Strep. faecalis*; – ⊘ –, *B. subtilis*; – ⊙ –, *Ps. denitrificans.*

suspensions of *Ps. denitrificans*, *B. subtilis* and *Strep. faecalis*. The release of 260 mμ. absorbing cellular material by suspensions of seven organisms treated with sufficient of the antibiotic to give complete saturation of the absorbing sites are recorded in Table 2. These results illustrate the considerable difference in behaviour exhibited by sensitive and resistant organisms.

Table 2. *Maximum amounts of* 260 mμ. *absorbing material released by bacteria in* 20 *min. at* 25° *in the presence of polymyxin E*

Suspensions in 0·01 M-phosphate buffer pH. 6·3.

Organism	260 mμ. absorbing material released (log I_0/I/mg. dry wt. bacteria/ml.)
Ps. denitrificans	0·46
M. lysodeikticus	0·92
Bact. coli	0·24
B. subtilis	0·40
Strep. faecalis	0·06
Staph. aureus	0·02
Pr. vulgaris	0·05

Effect of incubation time upon the absorption of polymyxin E and on the release of 260 mμ. *absorbing material*

In the preceding sections the absorption of polymyxin E by bacteria and its action in releasing cellular material were studied after an incubation time of 20 min. The effect of incubation time upon these two processes with *B. subtilis*, *Bact. coli* and *Strep. faecalis* are recorded in Table 3. Polymyxin E was added to suspensions of these organisms, and samples were withdrawn at intervals for the determination of polymyxin E absorbed and cellular material released. The bacterial suspensions were shaken by hand for *c.* 10 sec. at 10 min. intervals. With *B. subtilis* the absorption process was rapid and unaffected by incubation times from 10 to 250 min., whereas with *Bact. coli* and *Strep. faecalis* absorption of polymyxin increased progressively with time of incubation. However, with

Table 3. *Effect of time of contact upon the bacterial absorption of polymyxin E, and the release of* 260 mμ. *absorbing material*

Suspensions in 0·01 M-phosphate buffer (pH 6·3). A = 260 mμ. absorbing material (log I_0/I/mg. dry wt. bacteria/ml.); B = polymyxin E absorbed (μg./mg. dry wt. bacteria).

Organism		Time (min.)				
		10	30	70	170	250
B. subtilis, untreated	A	0·13	0·14	0·15	0·18	0·31
Polymyxin E treated (400 μg./ml.)	A	0·50	0·52	0·54	0·62	0·96
	B	310	310	310	303	303
Bact. coli, untreated	A	0·09	0·10	0·11	0·15	0·18
Polymyxin E treated (350 μg./ml.)	A	0·30	0·33	0·36	0·46	0·53
	B	142	147	174	186	218
Strep. faecalis, untreated	A	0·07	0·07	0·08	0·13	0·19
Polymyxin E treated (250 μg./ml.)	A	0·11	0·15	0·27	0·39	0·53
	B	62	76	99	130	142

the *Bact. coli* suspensions agglutination of the cells occurred on addition of the polymyxin E, and the dependence upon time of contact may in this case be due to the slow diffusion of the antibiotic into the agglutinated bacteria. Untreated suspensions of the three bacteria showed a steady release of 260 mμ. absorbing material with time of incubation. Addition of polymyxin E to suspensions of *B. subtilis* and *Bact. coli* caused an immediate increase in the amount of 260 mμ. absorbing material released, further material being released as incubation proceeded. Treatment of *Strep. faecalis* suspensions with polymyxin E caused very little immediate release of cellular material, but as incubation continued 260 mμ. absorbing constituents were released, as with the other organisms.

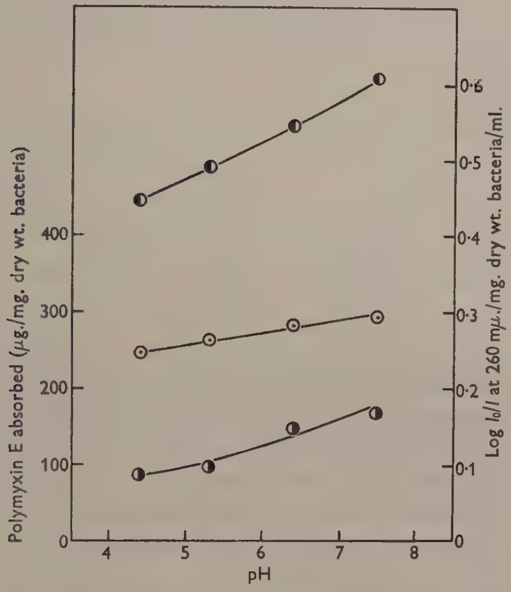


Fig. 4. Effect of pH value upon the absorption by *B. subtilis* of polymyxin E and the release of 260 mμ. absorbing material. Cells incubated at 25° for 20 min. in 0·01 M-acetate buffers (pH 4·4 and 5·3) and 0·01 M-phospate buffers (pH 6·5 and 7·6) alone, and in the presence of 500 μg. polymyxin E/ml. – ⊙ –, absorption of polymyxin E; – ◑ –, – ◐ –, release of 260 mμ. absorbing material from untreated and polymyxin-treated cells respectively.

Effect of pH *value upon the absorption of polymyxin E and on the release of* 260 mμ. *absorbing material*

Variation of the pH value of bacterial suspensions medium between pH 4·4 and 7·6 revealed that within this range no optimum pH occurred for the polymyxin E absorption process or for the cellular release mechanism. With *B. subtilis*, *Ps. denitrificans*, *Bact. coli* and *Strep. faecalis* a progressive increase occurred in the amount of polymyxin E absorbed, and 260 mμ. absorbing material released by both untreated and polymyxin E treated organisms when the pH of the suspension medium was between pH 4·4 and 7·6. The results obtained for *B. subtilis* are recorded in Fig. 4.

Relationship between the bactericidal action of polymyxin E and release of 260 mμ. absorbing material

The bactericidal action of polymyxin E and the release of 260 mμ. absorbing material was followed with suspensions of *Ps. denitrificans*, *Bact. coli* and *Strep. faecalis*. Samples were taken after 20 min. incubation at 25° for the estimation of surviving cells and release of cellular material; the results are shown in Table 4.

Table 4. *Bactericidal action of polymyxin E and release of 260 mμ. absorbing material*

Bacterial suspensions at 1 mg./ml. dry wt. in phosphate buffer (pH 6·3); samples taken after 20 min. incubation at 25°. A = log no. viable cells/ml. B = 260 mμ. absorbing material released (log I_0/I/ml.).

Polymyxin E concentration (μg./ml.)	*Ps. denitrificans*		*Bact. coli*		*Strep. faecalis*	
	A	B	A	B	A	B
·	9·48	·	9·20	·	9·72	·
5	9·18	0·08	8·85	0·04		
10	8·85	0·11	8·40	0·06	9·69	0·003
15	8·43	0·15	7·60	0·08		
20	7·40	0·19	6·78	0·10	9·69	0·01
25	4·80	0·24	4·48	0·13		
30	3·10	0·28	·	0·16	9·70	0·03

Absorption of polymyxin E by bacterial cell walls

The absorption isotherms of polymyxin E obtained with buffered suspensions of cell walls derived from *Ps. denitrificans*, *Bact. coli* and *Strep. faecalis* are shown in Fig. 5. As with the absorption isotherms obtained for the intact bacteria, a marked difference in behaviour was shown by cell-wall preparations derived from the polymyxin-sensitive *Ps. denitrificans* and *Bact. coli* when

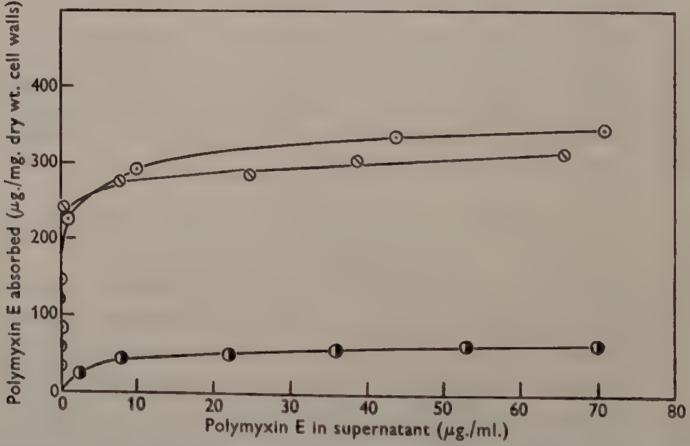


Fig. 5. Absorption of polymyxin E by bacterial cell walls. Cell walls were incubated at 25° for 20 min. in 0·01 M-phosphate buffer (pH 6·3) containing polymyxin E. – ◑ –, cell walls from *Strep. faecalis*; – ⊘ –, from *Bact. coli*; and – ⊙ –, from *Ps. denitrificans*.

compared with cell walls prepared from the resistant *Strep. faecalis*. Similar results were obtained with cell-wall preparations of the sensitive *B. subtilis* and the resistant *Staph. aureus*. No 260 mμ. absorbing cellular material was released by the cell-wall preparations on addition of polymyxin E.

Interfacial tensions of polymyxin E solutions

The interfacial tensions of buffered solutions of polymyxin E were investigated at the air/water and *n*-hexane/water interfaces. In Fig. 6 the depression of interfacial tension is plotted against the concentration of polymyxin E. At low polymyxin concentrations, the presence of the non-polar phase greatly facilitates orientation at the interface, whilst at concentrations greater than 20 μg./ml. further interfacial tension lowering occurs approximately at the same rate at both interfaces.

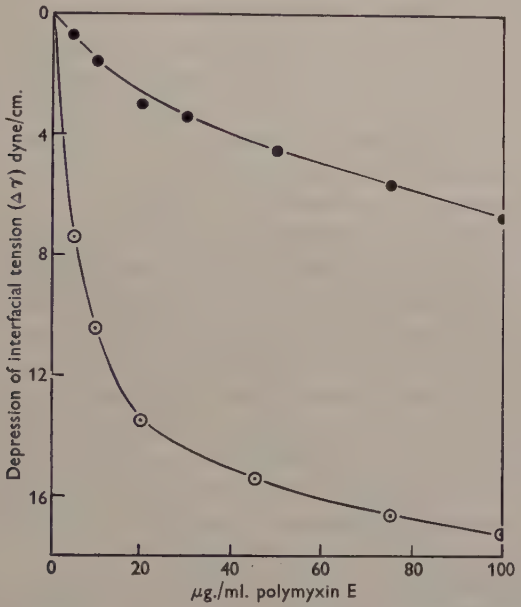


Fig. 6. Interfacial tensions of polymyxin E in 0·01 M-phosphate buffer (pH 6·3) at 20°. – ● –, air/water interface (interfacial tension 72·4 dynes/cm.); – ⊙ –, *n*-hexane/water interface (interfacial tension 51·0 dynes/cm.).

DISCUSSION

Polymyxin-sensitive bacteria have a high affinity for polymyxin. Addition of low concentrations of the antibiotic to suspensions of the four sensitive organisms studied resulted in almost complete absorption of the antibiotic upon the bacteria. At higher concentrations saturation of the organisms occurred and, with the exception of *Bact. coli*, uptake of the antibiotic was independent of the concentration of polymyxin E in the supernatant fluid. It appears that as a result of the agglutination of *Bact. coli* suspensions in the presence of the antibiotic the absorption process is impeded and complete saturation of the cells is not obtained even at high concentrations of polymyxin E. With this exception, the general absence of agglutination when cells are suspended in polymyxin E solutions even at saturation levels suggests that there is little absorption of the antibiotic upon the bacterial cell surface. This is in contrast to the agglutination of bacteria which occurs on treatment with CTAB observed by Salton (1951), although similar quantities of both compounds are absorbed by the bacteria. The shape of the absorption isotherms of polymyxin E with resistant bacteria shows that absorption is less complete at low concentrations of antibiotic, and that the saturation levels obtained after 20 min. contact with the antibiotic are much lower than those observed with the sensitive organisms. However, the amount of polymyxin absorbed at saturation levels by resistant and by sensitive organisms corresponds to more than that required for a closely packed monolayer of the antibiotic upon the bacteria. With the assumption that 1 mg. polymyxin E occupies an area of 0·80 sq.m., i.e. 160 A.^{2}/mol. (Few & Schulman, 1953*a*) the amount taken up at the saturation level by *Ps. denitrificans* would occupy an area of *c.* 12 × the cell surface area, assuming the cell dimensions to be 0·8μ. width and 2·5μ. length. The corresponding ratios for *Bact. coli* and *Strep. faecalis* are *c.* 10 × and *c.* 4 × the cell surface areas, assuming cell dimensions of 1μ. width, 2μ. length, and a sphere of 1μ. diameter, respectively.

The absorption isotherms obtained with cell walls derived from sensitive and resistant bacteria are similar to those obtained for the intact bacteria. Complete absorption of the antibiotic takes place upon cell walls prepared from sensitive bacteria on addition of low concentrations of polymyxin E, whilst absorption is incomplete upon cell walls derived from resistant bacteria. Assuming that the cell wall of the bacteria accounts for *c.* 15 % of the intact cell on a dry-weight basis (Few, Cooper & Rowley, unpublished observations; Mitchell & Moyle, 1951) the amount of polymyxin E absorbed by the cell walls at saturation levels corresponds to *c.* 1·8 × *c.* 2·3 × the cell surface area in the case of *Ps. denitrificans* and *Bact. coli*, respectively, and *c.* 0·4 × the cell surface area of *Strep. faecalis*. Thus, with the intact organisms, it is evident that at saturation levels polymyxin E absorbs at sites both upon the cell wall and inside the cell.

Addition of polymyxin E to suspensions of susceptible bacteria causes an immediate release of 260 mμ. absorbing material; more than 75 % of the material capable of release in 20 min. at 25° appears when the organisms are less than one-third saturated with polymyxin E. The amount of 260 mμ. absorbing material appearing in the supernatant fluid corresponds closely to that released on treatment of cells with sufficient CTAB to cause 99·99 % mortality (Salton, 1951). Salton also analysed chemically certain of the cellular constituents released by CTAB, and found that the amounts of inorganic and total phosphorus, pentose, glutamic acid, adenine and uracil were comparable to the quantities released when the cell suspensions were pipetted into boiling water. On treatment of sensitive bacteria with polymyxin E at concentrations less than those required for 99 % killing the amount of 260 mμ. absorbing material released in 20 min. is proportional to the percentage of cells killed. A similar relationship was observed by Gale & Taylor (1947) between the proportion of cells killed by different amounts of tyrocidin and the release of amino-acids, and by Salton (1951) between the proportion of cells killed by CTAB and the release of 260 mμ. absorbing material.

Complete saturation of susceptible organisms with polymyxin E is not necessary to render the cells non-viable. More than 99·5 % mortality is obtained in 20 min. when *Bact. coli* and *Ps. denitrificans* (at 1 mg. dry wt. bacteria/ml.) are treated with 20 and 25 μg. polymyxin E/ml. At these low polymyxin E concentrations the shape of the absorption isotherms of both intact bacteria and cell-wall preparations shows that complete absorption of the antibiotic takes place. Since the amounts of the antibiotic required for $>99{\cdot}5\,\%$ killing are much below the levels required for complete saturation of the bacterial cell wall it would be expected from the shape of these curves that the distribution of the antibiotic would be such as to favour absorption at sites within the bacterial cell wall, with but little penetration inside the cell. With this assumption, the amounts of polymyxin E required for *c.* 99·5 % killing approximate closely to those sufficient for the formation of a closely packed monolayer within the bacterial cell wall. Since, as a result of this absorption, there is a rapid release of soluble cellular constituents it is suggested that polymyxin combines with and also disorganizes, by a detergent-type action, the structures within the bacterial cell wall which are responsible for the maintenance of osmotic equilibrium. Once this has taken place there is both a diffusion of cell solutes into the external medium and, provided polymyxin E is present above the bactericidal concentration, a penetration of the antibiotic into the cell and reaction with intracellular components. The oil/water interfacial tension curve for polymyxin E shows that the antibiotic exhibits a strong tendency to absorb at hydrophobic interfaces. It has been pointed out by Hotchkiss (1946) for the case of synthetic surface-active agents that penetration of these substances into the cell would be expected to be appreciable only if accompanied by damage to the cell membrane. The results obtained here with polymyxin E support this general picture, the low and time-dependent absorption of the antibiotic by resistant bacteria being due to its poor penetration of the cell membrane.

It must be emphasized that the reactions of the surface-active antibiotics, and probably the synthetic detergents with the bacterial cell wall, would be expected to be specific interactions dependent not only upon the hydrophobic/hydrophilic balance and sterochemical arrangement of the polar groups present in the antibacterial agent, but also upon the nature of the structures within the bacterial cell wall. A similarity with these bacterial interactions is shown by the study of the mechanism of haemolysis by ionic detergents which possess identical non-polar groups (Pethica & Schulman, 1953). Haemolytic reactivity appears to depend upon detergency, or upon the lowering of interfacial tension, by complex formation with components present in the erythrocyte membrane, and is analogous to the ease of absorption and penetration by these agents of cholesterol monolayers at the air/water interface.

One of the authors (A.V.F.) wishes to thank the Medical Research Council for a personal grant.

REFERENCES

Baker, Z., Harrison, R. W. & Miller, B. F. (1941). Inhibition by phospholipids of the action of synthetic detergents on bacteria. *J. exp. Med.* **74**, 621.

Bliss, E. A., Chandler, C. A. & Schoenbach, E. B. (1949). *In vitro* studies of polymyxin. *Ann. N.Y. Acad. Sci.* **51**, 944.

Brownlee, G. & Bushby, S. R. M. (1948). Chemotherapy and pharmacology of aerosporin, a selective Gram-negative antibiotic. *Lancet*, i, 127.

Brownlee, G., Bushby, S. R. M. & Short, E. I. (1952). The chemotherapy and pharmacology of the polymyxins. *Brit. J. Pharmacol.* **7**, 170.

Dehéré, Ch. (1906). Sur l'absorption des rayons ultraviolets par l'acide nucléique extrait de la levure de bière. *C.R. Soc. Biol., Paris*, **60**, 34.

Few, A. V. & Schulman, J. H. (1953*a*). Unimolecular films of polymyxins A, B, D and E at the air/water interface. *Biochem. J.* **54**, 171.

Few, A. V. & Schulman, J. H. (1953*b*). Absorption of polymyxin by bacteria. *Nature, Lond.* **171**, 644.

Gale, E. F. & Taylor, E. S. (1947). The assimilation of amino-acids by bacteria. 2. The action of tyrocidin and some detergent substance in releasing amino-acids from the internal environment of *Streptococcus faecalis*. *J. gen. Microbiol.* **1**, 77.

Harkins, W. D. & Brown, F. E. (1919). The determination of surface tension (free surface energy), and the weight of falling drops: the surface tension of water and benzene by the capillary height method. *J. Amer. chem. Soc.* **41**, 499.

Hotchkiss, R. D. (1946). The nature of the bactericidal action of surface active agents. *Ann. N.Y. Acad. Sci.* **46**, 479.

Latterade, C. & Macheboeuf, M. (1950). Recherches biochimiques sur le mode d'action de la polymyxine. *Ann. Inst. Pasteur*, **78**, 751.

Mitchell, P. & Moyle, J. (1951). The glycerophospho-protein complex envelope of *Micrococcus pyogenes*. *J. gen. Microbiol.* **5**, 981.

Pethica, B. A. & Schulman, J. H. (1953). The physical chemistry of haemolysis by surface-active agents. *Biochem. J.* **53**, 177.

Rybak, B. (1950). Recherches sur la constitution du fuseau achromatique. 1. Études chimiques de quelques modèles protéiques. *Bull. Soc. chim. Biol., Paris*, **32**, 703.

Salton, M. R. J. (1951). The adsorption of cetyltrimethylammonium bromide by bacteria, its action in releasing cellular constituents and its bactericidal effects. *J. gen. Microbiol.* **5**, 391.

Salton, M. R. J. (1953). Studies on the bacterial cell wall. IV. The composition of the cell walls of some Gram-positive and Gram-negative bacteria. *Biochim. biophys. Acta*, **10**, 512.

White, H. J., Alverson, C. M., Baker, M. J. & Jackson, E. R. (1949). Comparative biological studies of polymyxin and 'aerosporin'. *Ann. N.Y. Acad. Sci.* **51**, 879.

(*Received* 29 *May* 1953)

KASSANIS, B. (1953). *J. gen. Microbiol.* 9, 467–474.

Some Effects of Sucrose and Phosphorus in increasing the Multiplication of Tobacco Mosaic Virus in detached Tobacco Leaves

BY B. KASSANIS

Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: Tobacco mosaic virus reached higher concentrations when inoculated tobacco leaves were placed in a solution containing 10 g./l. sucrose and 0·2 g./l. calcium phosphate than when in water. Detached leaves in water usually produced more virus than leaves left on the plants. Other sugars and phosphates also increased virus production. Sugar and calcium phosphate sometimes separately increased the concentration of the virus, but the response was usually greatest to both together. The increase varied with the nutritional state of the plants from which the leaves came and some other environmental conditions. Virus concentration, and the effect of sucrose and calcium phosphate in increasing it, was greater when leaves were in the light than in the dark. Conditions which increased virus concentration also increased the total carbohydrates of the leaves.

The effect of various substances on the multiplication of tobacco mosaic virus has been studied in detached tobacco leaves or in disks cut from the leaves. Most of such work has been done to find substances that inhibit multiplication, and there have been few experiments to find conditions under which the virus multiplies most extensively, although work with whole plants has demonstrated that host nutrition and environment both affect virus production. The experiments described below show that virus production is greater in detached leaves placed in solutions containing sugar and phosphate than in water, and that the extent to which sugar and phosphate affect virus multiplication varies with the physiological conditions of the leaves and depends on whether they are kept in the light or dark.

MATERIALS AND METHODS

The experiments were made with tobacco mosaic virus in tobacco (*Nicotiana tabacum* L. var. White Burley), using for inoculum a purified virus preparation at a concentration of 1 g./l. Quantitative infectivity tests were made by the local-lesion method, using *Nicotiana glutinosa* L. The tobacco leaves were detached from the plants a day after their inoculation and placed in distilled water or in solutions of the different substances being tested. This interval between inoculation and treatment was adopted to avoid the diminution in the number of local infections produced by floating leaves immediately after they are inoculated, a phenomenon that has also been described by Yarwood, Hall & Nelson (1953). Two to four tobacco plants provided the six to twelve leaves which were inoculated for every treatment; from these a total of 15–20 ml. of sap was extracted. The leaves were placed in shallow enamel dishes measuring 0·25–0·30 m. and containing a litre of the fluid; about half the total leaf surface was submerged. The dishes were covered with glass and, unless otherwise mentioned, kept in a glasshouse with a mean temperature of 20° for 5–8 days. Bacterial growth was checked by adding 0·3 g. sulphanilamide/l. fluid; this has no effect on virus multiplication, but usually kept the fluids clear and the leaves turgid until the end of the experiment. When the solution contained a nitrogenous substance it became turbid and the leaves slimy, but by changing the fluids after 3 days bacterial growth was kept below the level at which the leaves suffered. The floated leaves usually became chlorotic and sometimes developed local lesions, showing as green rings or spots against the yellow background of the leaf. The lesions appeared more often in leaves placed in nutrient solutions than in water.

At the end of the experiment the leaves were washed in tap-water, dried with a towel and ground in a mortar. The sap was extracted through muslin, heated at 60° for 10 min. and centrifuged. The virus was quantitatively isolated from 10 ml. of clarified sap by precipitating twice with 1/4-saturated ammonium sulphate solution and once at pH 3·3. The preparations were freed from salts by denaturing the virus with trichloroacetic acid, washing the denatured protein 3 times in distilled water and centrifuging. The protein was then dried in an oven and weighed. By this method 90 % of the virus present in the sap was recovered.

EXPERIMENTAL

Sucrose and phosphate

Table 1 records the results of three experiments to compare the concentration of tobacco mosaic virus in tobacco leaves placed for 5 days in water or in a nutrient solution containing 5 g. sucrose/l. and 0·2 g./l. each of NH_4NO_3 and $Ca(H_2PO_4)_2 . H_2O$. Exps. A and B had three replications and Exp. C two replications for each treatment. The leaves placed in the nutrient contained from 50 to 70 % more virus than the leaves in water. The replications differed little. Statistical analysis showed the difference to be significant between 1 and 0·1 % probability level and in Exp. C between 2 and 1 %. Although replications in individual experiments differed little, there were considerable differences in virus concentration with similar treatments in experiments done

Table 1. *The concentration of tobacco mosaic virus in leaves placed in water and in nutrient solution*

	Exp. A	Exp. B	Exp. C
Fluid	mg. virus/ml. sap		
Water	1·2	0·9	0·9
	1·3	1·1	1·0
	1·3	1·0	—
Nutrient solution	2·0	1·5	1·5
	2·2	1·4	1·6
	2·4	1·7	—
Difference between means	0·9	0·5	0·6
S.E. of the difference	0·120	0·105	0·071

at different times. In winter, for example, the inoculated leaves contained much less virus than in the summer; nutrient solution also had a proportionally greater effect on virus concentration in winter.

The concentration of virus depended on the amount of sucrose in the nutrient solution, and preliminary tests showed that most virus was usually obtained with 10 g. sucrose/l. in combination with 0·2 g. calcium phosphate/l. These concentrations were used throughout the rest of the experiments.

Of sucrose, NH_4NO_3, $Ca(H_2PO_4)_2 . H_2O$ present in the nutrient solution, only sucrose and calcium phosphate affected virus production, and whereas each separately often had little effect the two together usually produced a large response. To quote one experiment in which inoculated leaves were placed in water, a solution of calcium phosphate alone, of sucrose alone, and a mixture of the two, the amount of virus in sap was 0·5, 0·5, 0·6 and 1·1 mg./ml., respectively. In a few experiments, however, there was a good response to phosphate or sugar when given alone, and some of the conditions for such a response are described later. Potassium or ammonium phosphate behaved like calcium phosphate. Glucose, fructose, mannose, lactose and raffinose all increased the virus concentration to the same extent as did sucrose. Mannitol had a smaller effect and arabinose none.

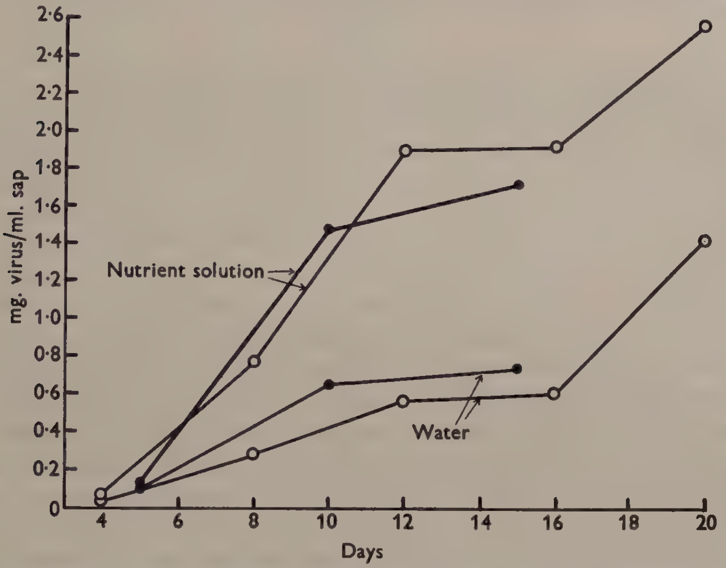


Fig. 1. The concentration of tobacco mosaic virus in sap (mg./ml.) from tobacco leaves placed in water or nutrient solution at different times from inoculations. Exp. A, ●—●; Exp. B, ○—○.

Nutrient solutions that greatly increased virus concentration when inoculated leaves were placed in them had little effect when leaves were left on the plant and sprayed with them. The mean yield of virus in sap from two replications in one experiment was (mg./ml.): leaves placed in water 0·7, placed in nutrient solution 2·1, leaves left on the plant and sprayed with water 0·4, leaves sprayed with nutrient solution 0·5. In this, as in seven out of fourteen experiments, sap from detached leaves placed in water contained more virus than sap from leaves left on the plant; in five of the other experiments there was no difference.

The difference in virus content between leaves placed in water and in nutrient solutions was maintained for longer than the 5–8 days for which most experiments ran. Fig. 1 gives results of two experiments in which inoculated leaves in water and in nutrient solution were sampled at different intervals up to 20 days after inoculation and shows that differences became apparent after 4 days and were maintained throughout. The leaves could not be kept longer, because, even with frequent changes of solutions, they became very chlorotic after 15–20 days.

Nitrogen

Virus multiplication was usually unaffected by the presence of a nitrogenous source in the solution, with or without sucrose and phosphate. In one typical experiment with solutions containing 10 g. sucrose/l. and 0·2 g. calcium phosphate/l., the mean yield of virus in sap for two replications was 1·45 mg./ml. with ammonium sulphate, 0·2 g./l. and 1·40 without it. In a few experiments the addition of nitrogen decreased virus concentration and in a few made in the summer increased it. Increases occurred with leaves taken from plants showing signs of nitrogen deficiency, and in such leaves virus production was increased by sucrose and phosphate only when nitrogen was added. The way in which the nutritional state of the plants at the time of inoculation influenced the effects of nutrient solution on the virus multiplication was shown by experiments with plants given different nutrients. Half of one group of nitrogen-deficient plants were given ammonium sulphate 1 g. per pot 2 weeks before inoculation. Half of the inoculated leaves in each group were placed in water and half in a solution containing sucrose and calcium phosphate. The concentration of virus in sap from the plants that received ammonium sulphate was (mg./ml.): 0·3 from leaves in water and 1·0 from leaves in solution; from the plants which did not receive ammonium sulphate 0·4 from leaves in water and 0·6 from leaves in solution.

Effect of light

Takahashi (1947) found that more tobacco mosaic virus was present in inoculated leaves detached and kept in the light than from those kept in the dark. The effect of light in stimulating virus production was confirmed in my tests with sucrose and calcium phosphate. Table 2 gives the results of one experiment in which the inoculated leaves, either left on the plant or detached, were kept either in light or in the dark, until harvest. Statistical analysis of the results showed that the virus concentration in leaves in nutrient solution was significantly higher ($P=1\%$) than that in leaves in water or on the plant. The concentration of virus in leaves in the light was significantly higher ($P=1–0·1\%$) than in leaves in the dark, but there was no interaction between light and nutritional treatment.

In this, and in other experiments when estimations were made for carbohydrates in expressed sap, leaves in nutrient solution contained more than those kept in water, and much more when in the light than in the dark. Iodine staining also showed considerable differences in the amount of starch formed in the leaves treated differently. The leaves in water had little starch when kept in the light and none in the dark, whereas leaves in the nutrient solution stained heavily when kept in the light, but only a band of cells at the end of the petioles stained heavily when leaves were in the dark. This difference in starch content was also obvious when the sap was centrifuged. Starch forms a distinct white zone in the sediment, and the size of this zone varied strikingly with the previous treatment of the leaves.

Table 2. *The effect of sucrose and phosphate on tobacco mosaic virus in tobacco leaves in the light and dark*

	Light			Dark	
Treatment of the leaves	Total carbohydrate in sap (mg./ml.)	Phosphorus in sap (μg./ml.)	Virus in sap (mg./ml.)	Total carbohydrate in sap (mg./ml.)	Virus in sap (mg./ml.)
Left on the plant	39	87	0·8	3	0·5
Placed in water	9	96	0·6	3	0·4
Placed in 10 g. sucrose/l. and 0·2 g. calcium phosphate/l.	23	274	1·1	8	0·6

All figures are means of two replications. The standard error of the concentration of virus is 0·067.

Phosphorus

Table 2 gives the phosphorus content of sap from the leaves in the light, and shows that these take up much phosphorus from the fluid. In other experiments, when phosphorus estimation was made on sap from leaves left in the light or dark, the amount in both was the same. That phosphorus is taken in quite readily was also shown by placing inoculated leaves in solutions in which KH_2PO_4 containing radioactive phosphorus was added. Indeed, this proved a better method of producing radioactive tobacco mosaic virus, than giving ^{32}P through roots. When tobacco plants are grown in water culture containing high concentration of ^{32}P their roots are damaged (Russell, Adams & Martin, 1949) so that the yield and radioactivity of the virus is lower than that from inoculated leaves placed in nutrient containing ^{32}P. From leaves placed in a nutrient containing 10 g. sucrose/l. and 300–400 μC of ^{32}P and 0·1 g. KH_2PO_4/l., tobacco mosaic virus was isolated 6 days later and had a specific activity of about 0·025 μC/mg. protein. On this occasion 40 g. leaves gave 30 mg. purified virus. The infectivity of the radioactive virus per unit weight was the same as that of virus isolated from leaves placed in water or leaves left on the plant.

Sucrose and phosphate usually interacted strongly in increasing the concentration of tobacco mosaic virus. In some experiments, however, one of the two substances was responsible for most of the increase. In one experiment with such a result, the pots of half of the plants were watered 5 times with 20 ml. of 5 mg. calcium phosphate/ml. before the plants were inoculated. The yield of virus in sap with the watered plants was (mg./ml.): 0·6 in water, 1·1 in sucrose, 1·1 in sucrose and calcium phosphate; for the unwatered plants it was 0·8, 1·3, 1·9, respectively, for the three treatments.

Other factors

Leaves from young and old plants responded equally well to sucrose and calcium phosphate. In one experiment the concentration of virus in sap was (mg./ml.): 0·7 and 1·4 for the young plants and 0·4 and 0·9 for the old plants, respectively, for leaves in water and in the nutrient solution. The higher virus concentration in young leaves probably occurred because inoculation produced more foci of infection. Virus production is also greater when inoculations are made with the aid of an abrasive, such as 'Celite', when the response to sucrose and calcium phosphate is also often proportionally greater than in comparable leaves inoculated without 'Celite'. Table 3 gives the results of two experiments on the effect of 'Celite' and of two experiments in which the inoculated leaves (without 'Celite') were kept continuously at 36° and compared with leaves at fluctuating glasshouse temperature with a mean of 20°. The leaves in water at 36° contained little virus, but phosphorus and sucrose had a proportionally bigger effect on virus content at 36° than at 20°. This probably occurred because the sucrose in the solution compensated for the carbohydrates lost by greater respiration at 36° than at 20°. At the end of Exp. A (Table 3) the total carbohydrates in crude sap for leaves were (mg./ml.): at 20°, 12·5 and 28·7, and at 36°, 6·9 and 28·6 respectively for water control and nutrient solution.

Table 3. *The effect of sucrose and phosphate on tobacco mosaic virus in leaves inoculated by different methods and left at different temperatures*

	Without 'Celite'		With 'Celite'		20°		36°	
	Exp. A	Exp. B	Exp. A	Exp. B	Exp. A	Exp. B	Exp. A	Exp. B
Treatment	virus mg./ml. sap							
Water	0·4	0·5	0·9	0·7	0·9	1·0	0·2	0·1
Nutrient containing 10 g. sucrose/l. and 0·2 g. calcium phosphate/l.	0·8	0·8	2·0	2·3	1·7	2·0	0·7	0·7

Yarwood (1952*b*) found that 31° was the optimal temperature for tobacco mosaic virus multiplication, and he could not detect virus in infected leaves kept at 37°. Although the virus does multiply at 36° (Table 3) this temperature is clearly far from optimal.

DISCUSSION

The results obtained with tobacco mosaic virus in tobacco plants may not apply generally to other viruses or hosts. Yarwood (1952*a*), testing the effect of sucrose on the multiplication of some viruses in inoculated leaves kept in the dark, found considerable differences between different virus-host combinations. His assays were made by inoculation to French beans and were expressed as lesions produced by extracts from equal weight of macerated leaf tissue. He found that tobacco mosaic virus in tobacco produced twice as many lesions in French beans when the inoculum was from leaves in 10% sucrose than in water, but has questioned the significance of this difference. With some other virus-host combinations he found different results. For example, the concentration of tobacco mosaic virus in French beans was higher in leaves placed in sucrose solution than in water, whereas tobacco ring spot virus in French beans gave the opposite results. Leben & Fulton (1952), searching for a method for screening the effect of different substances on viruses *in vivo*, found that tobacco necrosis and tobacco ring spot viruses produced lesions on detached cowpea leaves in the dark only when the medium supplied to the leaves contained sucrose.

In my experiments the leaves given sucrose not only had a greater virus content than in water but also accumulated considerable amounts of starch and other carbohydrates. This suggests that sucrose was not acting merely as a substrate for the energy required for virus multiplication but was generally stimulating cell metabolism. As protein synthesis can take place in the dark, provided there is a source of carbohydrate, it is rather unexpected that sucrose in the dark has a smaller effect than in the light. Analysis showed that the total carbohydrates were also low in leaves left in the dark, possibly because less sucrose was taken in by leaves in the dark than in the light. Phillis & Mason (1937) reported a similar observation; using disks of leaves previously destarched and immersed in solution of sucrose, no starch, or very little, was formed in the dark, whereas even diffuse daylight, insufficient for assimilation, promoted starch formation.

Placing detached leaves in the nutrient solution increased the virus concentration more than did spraying the solution on the leaves left on the plants. Less sucrose may enter the sprayed leaves, but detaching the leaves may also result in more favourable conditions for virus multiplication. Vickery *et al.* (1937) found that protein hydrolysis is stimulated by detaching tobacco leaves and proceeds rapidly both in light and dark for the first 72 hr. It is likely, then, that the virus finds the material for its synthesis ready-made in detached leaves.

The effect of phosphorus in increasing the multiplication of tobacco mosaic virus may simply be a consequence of the leaves usually containing less than the optimal amount. This is suggested by the results of one experiment in which plants were given extra phosphorus before inoculation. However, Bawden & Kassanis (1949), and Cheo, Pound & Weathers (1952), found that the concentration of tobacco mosaic and cucumber mosaic viruses, respectively, in plants was increased by increasing phosphorus to levels at which the growth of the plants was reduced.

I am indebted to Mr H. L. Nixon for measuring the radioactivity of the virus preparations containing ^{32}P.

REFERENCES

Bawden, F. C. & Kassanis, B. (1949). Some effect of host-plant nutrition on the multiplication of viruses. *Ann. appl. Biol.* **37**, 215.

Cheo, P. C., Pound, G. S. & Weathers, L. G. (1952). The relation of host nutrition to the concentration of cucumber virus 1 in spinach. *Phytopathology*, **42**, 377.

Leben, C. & Fulton, R. W. (1952). The effect of certain antibiotics on lesion production by two plant viruses. *Phytopathology*, **42**, 331.

Phillis, E. & Mason, T. G. (1937). On the effect of light and of oxygen on the uptake of sugar by the foliage leaf. *Mem. Cott. Res. Sta., Trinidad*, ser. B, no. 10.

Russell, R. S., Adams, S. N. & Martin, R. P. (1949). Radiation effects due to phosphorus—32 in fertilizer experiments. *Nature, Lond.* **164**, 993.

Takahashi, W. N. (1947). Respiration of virus infected plant tissue and effect on virus multiplication. *Amer. J. Bot.* **34**, 496.

Vickery, H. B., Pucher, G. W., Wakeman, A. J. & Leavenworth, C. S. (1937). Chemical investigations of the tobacco plant. VI. Chemical changes that occur in leaves during culture in light and in darkness. *Bull. Conn. agric. Exp. Sta.* no. 399.

Yarwood, C. E. (1952*a*). Some relations of carbohydrate level of the host to plant virus infections. *Amer. J. Bot.* **39**, 119.

Yarwood, C. E. (1952*b*). Latent period and generation time for two plant viruses. *Amer. J. Bot.* **39**, 613.

Yarwood, C. E., Hall, A. P. & Nelson, M. N. (1953). Nutritive value of rust-infected leaves. *Science*, **117**, 326.

(*Received* 6 *June* 1953)

RECORD, B. R. & TAYLOR, R. (1953). *J. gen. Microbiol.* **9**, 475–484.

Some Factors influencing the Survival of *Bacterium coli* on Freeze-drying

BY B. R. RECORD AND R. TAYLOR
Microbiological Research Department (Ministry of Supply), Porton, Wiltshire

SUMMARY: Quantitative studies have been carried out on the survival of bacteria on drying from the frozen state. Attention has been confined mainly to one species of vegetative bacteria, viz. *Bacterium coli*, of which several different strains were examined. When suspended in a phosphate buffer (pH 7·6) and dried from the frozen state (−35°), it was found that the percentage of organisms surviving varied widely with the strain used. With a given strain, it was found that a well-defined relationship existed between the concentration of organisms in the suspension before drying and the percentage of viable survivors recovered after reconstituting the dried product; the more dilute the suspension the lower the percentage of organisms surviving. When a low concentration of organisms was suspended in the cell-free filtrate obtained from a higher concentration of the same organisms, the percentage of viable organisms recovered approximated to the figure to be expected for the higher concentration of organisms. The dependence of percentage survival on the concentration of organisms in the suspension being dried was shown to be due to soluble material derived from the organisms themselves. On freezing alone, percentage survival was independent of the concentration of organisms in the suspension; the material affords protection during the drying stage of the process only. The protective effect of the material during freeze-drying was enhanced by the addition of glucose, especially during prolonged secondary drying.

The widespread development of freeze-drying apparatus and techniques in recent years has focused increasing attention on the preservation of bacteria by this means. The first real quantitative data on survival rates are to be found in the work of Stamp (1947), and this has since been followed by papers by Proom & Hemmons (1949) and Fry & Greaves (1951). The subject was briefly reviewed in Flosdorf's *Freeze-drying* (1949); various aspects are dealt with in the *Institute of Biology's Symposium on Freezing and Drying* (1951). Interest has so far centred on the practical problem of the preservation of organisms in a viable state in good yield and during storage for long periods, and work has been mainly directed to developing media and studying the conditions best fitted to achieve this goal. With the introduction of their '*mist. desiccans*', a serum broth glucose mixture, Fry & Greaves (1951) appear to have made a notable advance in this direction.

The present work represents a departure from this general approach in that studies of survival on freeze-drying have also been carried out in the absence of any protective substances other than any that may be supplied by the organisms themselves. Under these conditions, it was found that the percentage of organisms surviving drying increased with the concentration of organisms in the suspension being dried. It is further shown that the phenomenon is the result of the presence in the suspension of soluble material derived from the organisms themselves.

METHODS

Roux bottles containing tryptic meat broth were inoculated with a pure culture and incubated for 18 hr. at 37°. The broth was then transferred to 1 oz. screw-cap bottles and centrifuged at 1600 g. for 15 min. The supernatant was poured off and the residue of bacteria drained, after which, having been re-suspended in phosphate buffer, it was shaken in a small machine for 10 min. This washing process was repeated three times, further washings having been found to have no further effect on the results presented. The aim was to obtain a suspension entirely free from the growth medium, and this consideration dictated the choice of broth instead of nutrient agar for this purpose. After the third washing, the bacterial deposit was finally re-suspended either in water or phosphate buffer, as required, with 20 min. shaking (4·5 cm. stroke, 450 r.p.m.) to ensure the dispersal of any clumps, and to give a bacterial concentration in the range 10^9–10^{10} organisms/ml. The suspension was placed in the cold room (+2°) for 2 hr. before use. The phosphate buffer used throughout this work was the standard sterile buffer developed in this department for general bacteriological use, as a dilution fluid for plate counts, etc. It was made up (in %, w/v) from KH_2PO_4, 0·45; $(NH_4)_2\ SO_4$, 0·05; NH_4Cl, 0·05; adjusted to pH 7·6 by the addition of NaOH.

Serial dilutions were made in phosphate buffer with, usually, a tenfold dilution at each stage, except in the region where the final counts were made, when closer ratios were often used. Nine ml. volumes of phosphate buffer were placed in a set of 1 oz. screw-cap bottles, and 1 ml. of the suspension delivered into the first bottle from an accurate mark to mark pipette. After thorough mixing, 1 ml. of this diluted suspension was transferred (fresh sterile pipette each time) to the next 9 ml. volume of phosphate buffer, and so on until a suitable dilution for making the final counts was reached.

Counts were made by a modified Miles & Misra (1938) surface-culture method, 12 drops from each dilution being delivered from a calibrated dropping pipette (36 drops/ml.) on to each of six Petri dishes containing 30 ml. tryptic meat agar. The surface agar had been suitably dehydrated by pre-treatment in an incubator at 50° (1 hr. in the inverted position) such that the test 1/3 ml. delivered on the agar surface was absorbed into the agar in about 5 min. The colonies were counted after a suitable period of incubation at 37°.

The freeze-drying of suspensions

The Cryostat. This apparatus shown diagrammatically in Fig. 1 was designed primarily for the preparation by freeze-drying of histological specimens for microscopic examination. It was well adapted for the freeze-drying of small quantities (0·2 ml.) of bacterial suspensions, since the temperature throughout the drying process, a matter of considerable doubt with the usual types of freeze-drying equipment, could be controlled precisely. The part of the apparatus containing the ampoules was immersed in a bath of acetone contained in a 1 gal. thermos jar, and refrigerated by a copper coil connected via an automatic regulator to a refrigerator compressor. The bath temperature was maintained within a degree or so of any desired temperature by a Sunvic thermostat which switched the compressor on and off via an inductive hot-wire relay. By using two domestic-type refrigerator units in cascade, bath temperatures down to −65° could be obtained using Freon 12 as a refrigerant. The 0·2 ml. samples contained in small tubes, and held in a rack, were rapidly frozen by dipping in the acetone bath for a few minutes. They were then transferred to the Cryostat, which had already been cooled to the desired drying temperature. The apparatus was evacuated to about 1 μ. Hg pressure by means of an Edwards two-stage rotary oil pump, pressure being read on a sensitive McLeod gauge. Moisture was absorbed from the system by phosphorus pentoxide contained in a tray; such an absorbent is satisfactory for the small quantities of material being handled. At no time during the drying process can the temperature of the product exceed that of the bath, which therefore represents the maximum temperature of drying and, in fact, the temperature at which the final stages of the drying process are carried out. For very small amounts of material, an apparatus employing a refrigerated chamber is probably essential if true freeze-drying is to be achieved. Stamp (1947) described freeze-drying at a controlled temperature (−23°) by means of the McFarlane process.

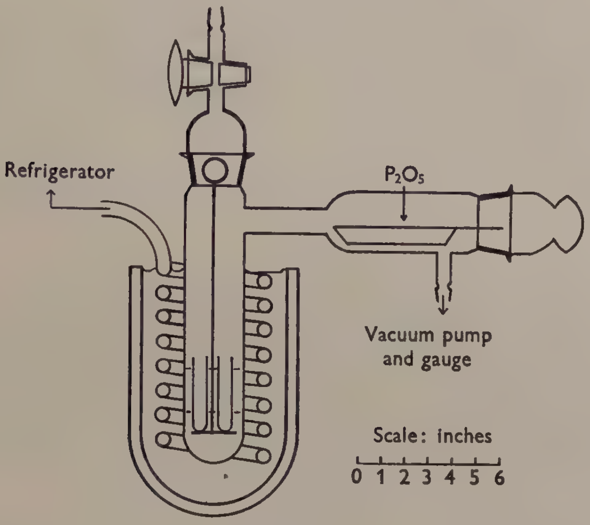


Fig. 1

It was found that the survival rates obtained under these conditions at temperatures down to −50° were not significantly different from those obtained using the department's standard equipment in which the low temperature of the drying mass is maintained by virtue of its own evaporation. Most of the present work has been carried out on this standard equipment, where larger volumes could be handled more conveniently and quickly.

The M.R.D. freeze-drying apparatus. This equipment, designed by one of us (B. R. R.) and built in the department's workshops, has not before been described in the literature, so a brief account will be given here.

The design has been based on the vacuum spin-freeze principle introduced by Greaves (1944), and was adopted in the first place to deal with the problem of freeze-drying from 3 lb. Kilner bottling jars (250 ml./jar) without the necessity for pre-freezing. The first machine accommodated eighteen of these jars, with the provision for vertical spinning of each jar about its own axis in the vacuum chamber, by means of a train of gears. The satisfactory results obtained with this machine led us to construct a second machine to accommodate sixty of the 1 oz. screw-cap type of bottle, each bottle being rotated at 2000 r.p.m. about its own vertical axis, by gear drive from a central shaft, driven from a motor external to the vacuum chamber.

This departure from the usual practice of placing the ampoules or bottles in inclined holes drilled in a large rotor, while no longer as essential as with the larger size of bottle, has the merit of distributing the contents of each bottle in a thin film of approximately uniform thickness round the entire wall of the bottle, instead of in the form of a wedge at a lower corner. Much more rapid and uniform drying is thereby achieved, 10 ml. quantities being dry in about 4 hr. without any heat being applied.

Pl. 1, fig. 1, shows the external view of the vacuum chamber. The refrigerated coil occupying the lower part of the chamber is maintained at $-45°$ by a 1 H.P. compressor, located behind the panel in a sound-proof compartment. This also contains the vacuum pump (Edwards, 2S 150) of the two-stage rotary oil type. Pl. 1, fig. 2, shows an interior view of the upper half of the chamber, with the sixty gear-driven containers. The temperature of a representative sample is recorded during the drying process by means of a fine wire thermocouple connected to a copper:constantan slip ring assembly fixed to one of the containers, as seen in the illustration.

The bottles are spun for the first 20 min. or so only, by which time the vacuum has reached about 0·1 mm. Hg and the product temperature $-25°$, after which the spinner motor can be stopped and the drying allowed to proceed in the usual manner. With a coil temperature of $-45°$, the product assumes a temperature of about $-40°$ with uncapped bottles, or about $-35°$ when aseptic precautions are taken by the fitting of sterile cotton-wool and gauze caps, as in the present work. From the chart recording product temperature, it appears that freezing is practically instantaneous, there being no evident break in the curve at any point. An appreciable part of the water in the solution is evaporated in the initial stage of the process in withdrawing heat to freeze and to cool the remainder down to $-35°$. A further quantity is lost in cooling the walls of the bottle down to this temperature and for this reason it is not advisable to use too small a volume of solution per bottle if a true freeze-dry is to be ensured. Most of this work was carried out with 5 ml./bottle which provides an ample margin. If the volume greatly exceeds 15 ml. loss of liquid occurs from the neck of the bottle during the spinning stage.

While primary drying is complete in about 4 hr., we have, as a routine in this work, continued the process overnight so that all samples have had a period of 24 hr. in the primary chamber. The results obtained after this treatment are referred to as the survival on primary drying only. In a number of cases we have continued the drying of the primary dried product by storing in a vacuum of $<0\cdot01$ mm. Hg at room temperature over phosphorus pentoxide.

In quantitative work involving freeze-drying it is necessary to be on guard in case material should be ejected from the bottle during the drying process—the so-called 'spill' (see, for example, S. T. Cowan, p. 127, and Wilson Smith, p. 139, of the *Inst. Biol. Symp.* 1951). This is especially likely to occur with dilute salt solutions, or with particle suspensions in water or dilute salt, where the finely divided dried layer on the inside appears to be carried away by the high-velocity vapour stream emerging from beneath it. Sodium chloride is notorious in this respect, nearly half being ejected during the freeze-drying of a 1 % solution from an open-necked bottle. The phosphate buffer used for the present work is by no means exempt, losses of 10–20 % being found with open bottles. With the gauze/cotton-wool drying caps on the bottles, however, gravimetric determinations showed no significant loss of phosphate buffer from the bottle under the conditions used. (With 1 % NaCl a heavy deposit piles up on the underside of the cap.)

RESULTS

Comparison of six different strains of coliform bacteria

Suspensions containing approximately 10^6 orgs./ml. in phosphate buffer were freeze-dried. The survival figures after primary drying are given in Table 1.

Table 1. *Survival of various strains* (*approx.* 10^6 *orgs./ml. in phosphate buffer*) *after freeze-drying*

Strain	Viable survivors (%)
Bact. coli communis	1·4
Bact. coli 'Jepp'	0·2
Bact. aerogenes (NCTC 418)	0·17
Bact. coli '1100'	0·06
Bact. coli (NCTC 4450)	0·01
Bact. coli American Type B	0·006

It is seen that the survival under given conditions depended to a great extent on the strain of organism used. The most sensitive 'American type B' and a fairly robust strain 'Jepp' were selected for more detailed study.

The effect of cell concentration on survival

The question of cell concentration has been largely ignored in studies on the effect of freezing, and freeze-drying on viability. Stamp (1947) asserted that in the drying of *Streptococcus pyogenes*, *Pasteurella pestis* and *Salmonella typhimurium* in nutrient gelatin + ascorbic acid, the percentage survival rate rose with a diminution in cell concentration. The opposite effect was reported by

Otten (1930) who dried various pathogenic bacteria in saline at reduced pressure in a desiccator over H_2SO_4. Fry & Greaves (1951) were unable to demonstrate any significant difference in the survival rate of paracolon D 20 IM over the range $4{\cdot}7 \times 10^9$ to $4{\cdot}7 \times 10^4$ orgs./ml. when freeze-dried in their '*mist. desiccans*'. In the absence of any protective medium, we found that the concentration of organisms in the suspension being dried had a very marked effect on the percentage surviving freeze-drying, the survival rate falling with diminishing cell concentration. The experimental points in Fig. 2 were obtained from three independent experiments, with *Bact. coli* 'Jepp' freeze-dried in phosphate buffer. The curve is drawn to indicate the general trend.

Fig. 3 shows a similar series of results obtained with the fragile 'American B' strain of *Bact. coli*. In this case there were no survivors immediately after freeze-drying a 10^4 orgs./ml. suspension.

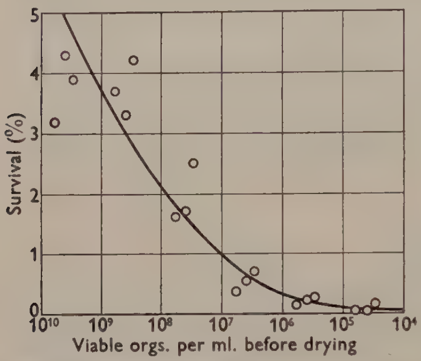


Fig. 2. Freeze-drying of *Bact. coli* Jepp in phosphate buffer. (24 hours drying).

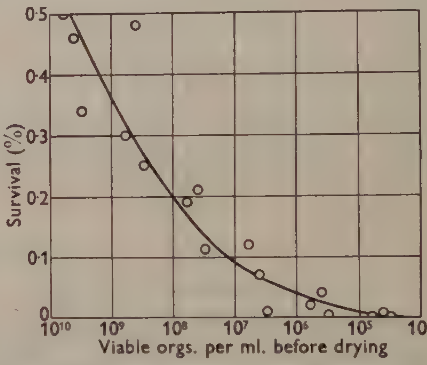


Fig. 3. Freeze-drying of the American 'B' strain of *Bact. coli* in phosphate buffer.

It appears at first sight that the surviving organisms are protected by the proximity of their moribund neighbours, and indeed, Otten (1930) had already shown that a killed suspension of the same or even of a different organism had a protective action on living organisms dried in saline under the conditions used by him (reduced pressure over H_2SO_4). In the absence of clumping, however, such a hypothesis is untenable in the case of true freeze-drying since in the completely frozen condition, below the eutectic point of the suspending medium, each organism is fixed firmly in position out of range of its neighbours. It becomes necessary to postulate, therefore, the presence of some material derived from the organisms themselves, and already in solution before freeze-drying commenced, and it seemed reasonable to suppose that the concentration of this material present in solution would depend on the concentration of organisms in suspension, thus explaining the concentration/survival relationship observed.

The effect of the cell-free supernatant on survival

The presence of a soluble protective substance was readily demonstrated by taking the cell-free supernatant (obtained by centrifuging and filtering through a sintered glass filter) from a 10^{10} orgs./ml. suspension of *Bact. coli* 'Jepp' in phosphate buffer, the suspension having been made up 2 hr. before. A 10^4 orgs./ml. suspension of the same organism when freeze-dried from the supernatant showed a survival of 5·7 % as against a value of about 0·2 % for a 10^4 orgs./ml. suspension in phosphate buffer only. In fact, the 10^4 orgs./ml. suspension now showed the same survival as that for a 10^{10} orgs./ml. suspension.

It is evident, therefore, that the organisms release, either by secretion or by the lysis of a small fraction of their number, a substance which, even in very low concentrations, has a remarkable effect in protecting viable cells against damage during freeze-drying.

Preparation of the bacterial exudate from Bact. coli *'Jepp'*. By freeze-drying an aqueous suspension of *Bact. coli,* reconstituting with water, and freeze-drying a second time, a product was obtained practically devoid of living cells (0·00001 % survivors). This contained a large proportion of the bacteria in solution, e.g. a 10^{10} orgs./ml. suspension of well-washed *Bact. coli* 'Jepp' treated in this manner, gave after centrifuging down the cell debris (and Seitz filtration), a solution containing 0·15 % soluble material. This was dispensed in screw-capped bottles and stored in the freeze-dried state.

The effect of the bacterial exudate on survival. The freeze-dried bacterial exudate thus obtained was taken up in phosphate buffer and serially diluted in tenfold steps. A freshly washed suspension of *Bact. coli* 'Jepp' was added to each tube to give a final concentration of approximately 10^4 viable orgs./ml. This is a convenient concentration of organisms for exploring the effect of any additive on survival, there being usually sufficient survivors to count with fair accuracy without at the same time sufficient organisms present to influence the result to any extent. The results are given in Table 2.

Table 2. *The survival of* Bact. coli *(Jepp)* (10^4 *orgs./ml.*) *after freeze-drying in varying concentrations of* Bact. coli *exudate*

Concn. exudate (g./100 ml.)	Survival (%)
0·30	12·6
$0{\cdot}30 \times 10^{-1}$	8·7
$0{\cdot}30 \times 10^{-2}$	4·0
$0{\cdot}30 \times 10^{-3}$	1·4
$0{\cdot}30 \times 10^{-4}$	0·66
$0{\cdot}30 \times 10^{-5}$	0·34
$0{\cdot}30 \times 10^{-6}$	0·24
Control: phosphate buffer only	0·21

The effect of prolonged secondary drying

When the freeze-dried suspensions of *Bact. coli* 'Jepp' in phosphate buffer were subjected to extended and vigorous drying over P_2O_5 under a vacuum of 0·01 mm. Hg or less, the fall in viability was rather rapid. Fig. 4 shows the general trend; after 21 days secondary drying a 4×10^8 suspension contained only 0·1 % survivors, while a 4×10^7 suspension had no survivors.

When dried in the presence of the prepared *Bact. coli* exudate (approx. 0·05 %) more stable conditions obtain, e.g. a 10^8 orgs./ml. suspension of *Bact.*

coli 'Jepp' which had a survival after primary drying of 20 % showed a viability of 4 % after 7 days drying, 2·8 % after 21 days and 2·1 % after 53 days. It was not, however, until the addition of glucose (Fry & Greaves, 1951) that any real stability was obtained. Thus, in the presence of *Bact. coli* exudate (0·25 %) and 5 % glucose the figures shown in Table 3 were found for *Bact. coli* 'Jepp'. The sensitive 'American B' strain of *Bact. coli* dried under the same conditions showed some fall in viable count in the initial stages, but settled down to a figure of about 10 % survival.

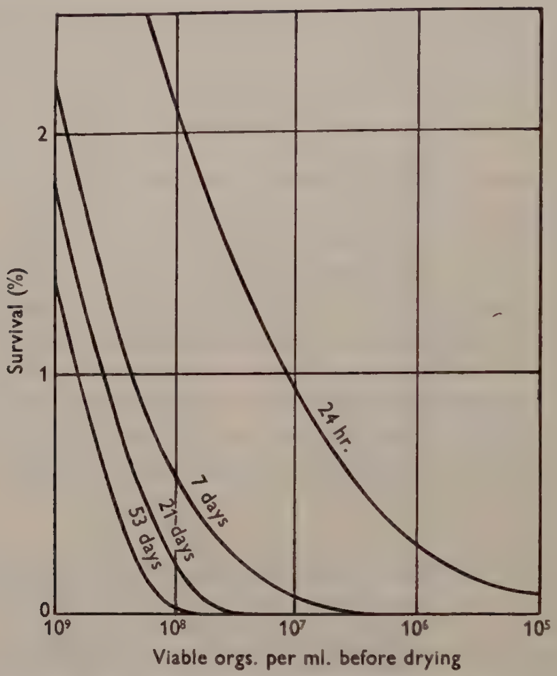


Fig. 4. Freeze-drying of *Bact. coli* Jepp in phosphate buffer. Prolonged secondary drying.

Table 3. *Survival of* Bact. coli *'Jepp' after secondary drying of suspensions containing* Bact. coli *exudate* (0·25 %) +*glucose* (5 %)

Concn. of organisms before drying (orgs./ml.)	Survival after secondary drying for (days)					
	1	16	30	59	188	330
	Survival (%)					
1×10^8	51	44	41	37	41	38
1×10^4	42	39	33	36	34	35

DISCUSSION

The role played by the concentration of organisms in a suspension in determining the resulting survival after freeze-drying has been investigated in detail so far only in the coliform group of bacteria, though we have found a similar though less pronounced effect in the case of *Chromobacterium prodigiosum* (*Serratia marcescens*), and the absence of a gradient with *Corynebacterium xerosis*. Independently, Hutton & Shirey (1951) observed a similar effect with *Brucella abortus*, though only over a very limited range of concentration. It would appear that the phenomenon may be expected to occur with any species of organisms which are easily disrupted and release the protoplasmic contents of some of their cells into solution. The phenomenon is of course masked in the presence of a medium with very strong protective properties, and it is not therefore surprising that Fry & Greaves (1951) failed to find any concentration effect in the case of bacteria dried in their '*mist. desiccans*'. Even cultures of *Bact. coli* grown on nutrient agar tend to give variable results owing to the presence of free particles of agar in the washed suspensions.

The effect of the age of a suspension on the percentage survival has not been investigated, but it appears probable that the concentration of material in solution would increase with the duration of storage and so result in higher survival rates, always supposing that the viable cells surviving still retain their original robustness. The initial shaking of the suspension, an essential step towards securing a dispersion of single organisms free from clumping, doubtless has a similar effect in breaking down the weaker cells and in raising the concentration of material in solution. The effect of shaking on the destruction of *Bact. coli* is dealt with in detail in a recent paper by Furness (1952).

Since freezing is an inseparable preliminary part of the freeze-drying process, it was of interest to examine the effect of bacterial concentration and of added bacterial exudate in protecting organisms against death on freezing alone. A single rapid freeze (−78°) and thaw of a *Bact. coli* 'Jepp' suspension in phosphate buffer resulted in a 40 % loss of viable organisms regardless of the concentration of organisms in the suspension over the range from 10^{10} to 10^4 orgs./ml. and was not appreciably affected by the addition of bacterial exudate. It therefore appears that the exudate offers no protection against damage by ice crystal formation, concentration of salts, etc., but is effective only during the drying stage of the freeze-drying process.

Whether the protective action during the drying stage is due to the formation of a layer of colloidal material which reinforces the fragile cell envelope, to the retention of a certain minimal amount of moisture in the cell or to other causes is not at present understood. That free moisture is in fact essential to survival would appear open to question in view of the results obtained in the presence of coli 'extract' with the addition of 5 % glucose, where negligible loss in viability occurred after nearly one year of continuous rigorous drying (see Table 3). If moisture is essential, clearly the amount required is infinitesimal.

The physico-chemical properties of the bacterial exudate have only been briefly investigated. It passes very slowly, if at at all, through a cellophan membrane; after 4 days dialysis at 2°, the presence of protective material in the dialysate was still in doubt; from its behaviour in the ultracentrifuge it appears to be very polydisperse. Its protective effect is not specific for the organism from which it is prepared, e.g. the material obtained from *Bact. coli* offered substantial protection to *Chr. prodigiosum*. Whether the material, or some component of it, has any property which makes it unique in the field of bacterial preservation remains still to be assessed.

Of chief importance perhaps is the realization of the existence of the phenomenon described, namely that under adverse conditions, such as the freeze-drying of aqueous suspensions of organisms, survival may depend to a large extent on their concentration, the survivors deriving protection from a material supplied by the organisms themselves, and a degree of protection proportional to the concentration of this material.

The authors wish to record their debt to Dr D. W. Henderson for his encouragement and advice. Acknowledgement is made to the Chief Scientist, Ministry of Supply, for pemission to publish. Plate 1, Crown Copyright reserved, is reproduced by permission of the Controller, H.M.S.O.

Patent Application No. 19892/53 for the M.R.D. Freeze-drying apparatus was filed on 17 July 1953.

REFERENCES

Flosdorf, E. W. (1949). *Freeze-Drying.* New York Rheinhold Publishing Co.

Fry, R. M. & Greaves, R. I. N. (1951). The survival of bacteria during and after drying. *J. Hyg., Camb.* **79**, 220.

Furness, G. (1952). Some factors affecting the destruction of *Bacterium coli* by shaking with Glass Beads. *J. gen. Microbiol*, **7**, 335.

Greaves, R. I. N. (1944). Centrifugal vacuum freezing. *Nature, Lond.* **153**, 485.

Hutton, R. S. & Shirey, W. N. (1951). Evidence that suspensions of *Brucella abortus* contain a substance which influences the degree of killing caused by freeze-drying. *Bact. Proc.* (*51st gen. Meeting*), p. 34.

Institute of Biology (1951). *Report on Symposium Freezing and Drying.* London.

Miles, A. A. & Misra, S. S. (1938). Estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

Otten, L. (1930). Die Trockenkonservierung von pathogenen Bakterien. *Zbl. Bakt.* (1. *Abt. Orig.*), **116**, 199.

Proom, H. & Hemmons, L. M. (1949). The drying and preservation of bacterial cultures. *J. gen. Microbiol.* **3**, 7.

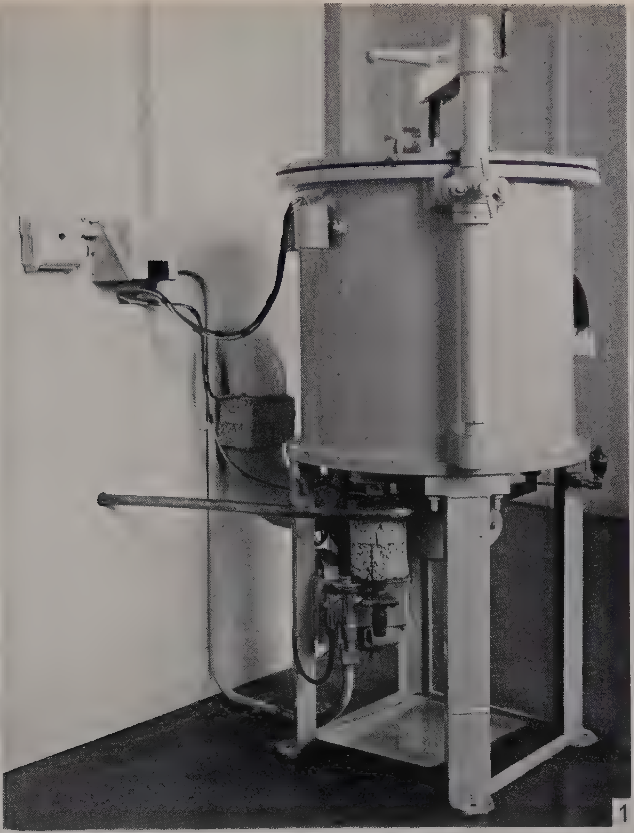
Stamp, Lord (1947). The preservation of bacteria by drying. *J. gen. Microbiol.* **1**, 251.

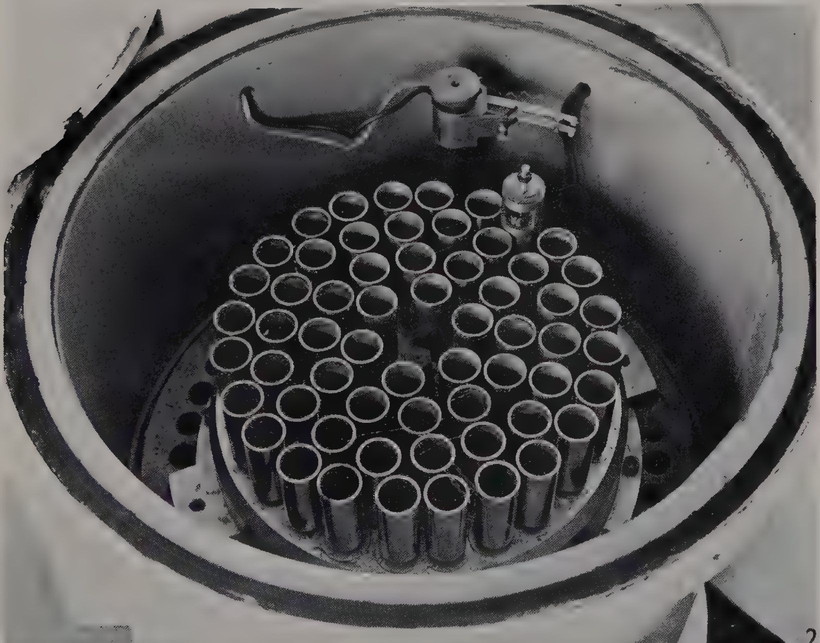
EXPLANATION OF PLATE

Fig. 1. External view of vacuum chamber. The compressor for the −45° refrigerated coil situated in the lower part of the chamber, and the Edwards two-stage rotary oil type vacuum pump are located behind the panel on the left in a soundproof compartment.

Fig. 2. A view of the interior of the upper half of the chamber showing the sixty gear-driven containers. The fine-wire thermocouple for temperature recording during the drying process is shown fixed in one of the containers.

(*Received* 6 *June* 1953)





B. R. RECORD AND R. TAYLOR—THE SURVIVAL OF *BACT. COLI* ON FREEZE-DRYING. PLATE 1

Baker, H., Sobotka, H. & Hutner, S. H. (1953). *J. gen. Microbiol.* 9, 485–493.

Growth Requirements of some Thermophilic and Mesophilic Bacilli

By H. BAKER and H. SOBOTKA

Department of Chemistry, The Mount Sinai Hospital, New York 29, N.Y., U.S.A.

and S. H. HUTNER

Haskins Laboratories, New York 17, N.Y., U.S.A.

SUMMARY: To approach reproducibility of mass cultures as sources of enzymes, the growth requirements of two mesophils belonging to *Bacillus licheniformis* and *B. circulans*, and three thermophils belonging to *B. licheniformis*, *B. circulans* and *B. stearothermophilus*, were determined in metal-buffered media.

The mesophilic *B. licheniformis* required glycerol or glucose, also alanine, aspartate, glycine, glutamate, arginine, histidine and lysine. Cytidylic acid or guanylic acid further increased growth; adenosine was inactive. The thermophilic *B. licheniformis* required for good growth either glycerol, gluconate, or glucose, also succinate or glutamate; glutamate was replaceable by the combination of arginine + histidine + proline. In glycerol media, either DL-malate alone, or citrate plus either acetate or fumarate supported growth; neither acetate nor fumarate were effective by themselves. In the absence of intermediates of the citric acid cycle, sodium bicarbonate or a combination of protogen + acetate allowed growth. With acetate present, protogen was replaceable by thiamine.

The *B. stearothermophilus* strain utilized glucose or sucrose; thiamine, biotin and nicotinic acid were required. A combination of arginine + histidine + isoleucine increased growth. The carbohydrate requirement appeared absolute.

The thermophilic *B. circulans* required glutamate or a combination of succinate + arginine + histidine; these sufficed for moderate growth. Supplementation with glycerol, sucrose, gluconate or glucose increased growth. Further addition of acetate decreased the lag phase. For the mesophilic *B. circulans*, either glucose or gluconate were suitable substrates; thiamine, biotin, riboflavin, and 'reduced sulphur' were required.

The advantages of thermophilic bacteria for studies of enzyme turnover are defined in recent reviews (Clegg & Jacobs, 1953; Allen, 1953). Before testing the thermostability of enzymes in thermophilic bacteria, chemically-defined culture media suitable for mass cultures were needed to ensure good yields of organisms and enzymes, and reproducibility of enzymic activities. Such media were especially desirable for work with proteolytic enzymes whose activities are conspicuously dependent upon Mn, Mg, Co, Ca or Zn ions (Smith, 1951; Crook, 1951). The concentration of these ions in natural materials varies widely.

Knight & Proom (1950) noted that many strains of *Bacillus circulans* grew poorly in defined media suitable for strains of other species. One of our mesophils and one of our facultative thermophils belonged to the *B. circulans* group, and it was therefore of interest to extend the study of Knight & Proom. The basal medium devised for this purpose appears suitable for mass cultures of our *B. circulans* strain and also for mesophilic and thermophilic variants belonging to several species and for a facultatively thermophilic strain of *B. stearothermophilus*—the species which includes the obligately thermophilic bacilli—since the technique devised for this purpose was simple, and the information so obtained was readily applicable to the mass cultivations needed for isolating enzymes in quantity. The definitions employed here for degrees of thermophily are those of Clegg & Jacobs (1953).

MATERIALS AND METHODS

The culture methods were essentially those described elsewhere for aerobic protists (Hutner, 1950; Hutner, Provasoli & Filfus, 1953). Experimental media in 5 ml. amounts were distributed in 25 ml. Pyrex Erlenmeyer flasks provided with glass or aluminium caps. In later work, to conserve space, media were distributed in 5 ml. amounts in 10 ml. borosilicate flasks having a broad base, thus resembling Fernbach flasks in shape; these flasks (Kimble Glass Company, Toledo, Ohio) allowed a greater surface : volume ratio (i.e. better oxygenation), and the flasks were less top-heavy. The flasks were incubated within chambers formed by inverting one Pyrex tray over another and sealing the joint with cellulose tape. Each chamber held up to thirty-eight 10 ml. flasks. Water was added to each tray to minimize the hazards of evaporation and uneven temperatures which are intensified at the incubation temperature for the thermophils. The temperatures of incubation, 37° for the mesophils and 55° for the thermophils, were read from thermometers kept in uninoculated flasks of culture medium; the difference between the air temperature in the incubator and that of the cultures was less than 0·5°. Maximum growth for all cultures usually required 5–7 days.

All stock cultures were maintained on: Nutri-Peptone (Baltimore Biological Laboratory), 0·5 g.; beef extract (Difco), 0·5 g.; agar, 2·0 g.; distilled water to 100 ml.; pH 6·5–7·0. Subcultures were made monthly and stored in the refrigerator after incubation. Bacteria scraped from agar slopes and suspended in the basal medium served as inocula. Growth was measured in optical density (O.D.) units as determined with a Welch Densichron. An O.D. of 1·0 equalled 0·50–0·55 g. dry-wt. washed bacteria/litre.

The basal medium shown in Table 2, when supplemented with appropriate substrates and growth factors, allowed heavy growth of the organisms listed in Table 1; this medium could be made in double strength and refrigerated without precipitation.

The concentrations of metals listed in Table 2 refer to the metal content of the salts used; these were usually sulphates rather than the hygroscopic chlorides. Calcium was prepared as $CaCO_3$ dissolved in HCl. Molybdenum salts are poorly soluble in acid solutions and were not combined with other metals in one acidic stock solution. To avoid precipitation the molybdenum stock solutions were adjusted to pH 8·0–8·5 with alkali.

*Trans*aconitic acid + triethanolamine served as buffers. Under the conditions of these experiments there was no evidence that this buffer pair had substrate activity. The pronounced alkalinization of the media (the pH may rise above 8·5) resulting from the consumption of organic acids and the liberation of ammonia from the amino-acids limited growth, making the use of concentrated buffer desirable.

Table 1. *Species of* Bacillus *used*

Strain	Source
Mesophil	
B. licheniformis, no. S8 (Ford strain)	I. M. Gunsalus
B. circulans	ATCC, no. 7046
Thermophil	
B. licheniformis, no. S8	Thermophilic variant of the Ford strain isolated by M. B. Allen
B. circulans, no. 7–2	Isolated from soil by M. B. Allen
B. stearothermophilus, no. B1102	Originally from the Northern Regional Laboratory; sent through the courtesy of W. G. Walter.

Table 2. *Basal medium*

Ethylenediamine tetraacetic acid (EDTA)	0·05 g.	NH_4Cl	0·12 g.
KH_2PO_4	0·02 g.	Ca (as Cl)	0·001 g.
$MgSO_4.7H_2O$	0·05 g.	*Trans*aconitic acid	0·5 g.
Mo (as ammonium molybdate)	0·001 g.	Triethanolamine	1·0 g.
'Metals 42'	1·5 ml.	Distilled water to	100 ml.

EDTA was used as the free acid purified by several recrystallizations from water; this preparation was a gift from the Bersworth Chemical Co., Framingham, Mass. The pH value of the medium was adjusted with KOH or H_2SO_4. The initial pH of the medium was 6·0–7·0 depending on the tolerance to the lower pH limit of the organism. One ml. of 'Metals 42' contains (mg./ml.): EDTA, 2·5; Zn, 4·0; Mn, 3·0; Cu, 0·25; Fe, 0·1; B (as H_3BO_3), 0·1; Co, 0·04.

RESULTS

Bacillus circulans—*mesophilic strain*

Substrates. Initial pH 6·0. Glucose or gluconate (0·5%; % means % w/v, throughout unless otherwise indicated) were equally effective as substrates. Gluconate offered the advantage of being obtainable in pure form from the δ-gluconolactone. The excess of chelating metals in the basal medium helped to compensate for the metal-binding effect of gluconate; the effectiveness of gluconate had been overlooked in previous experiments because of the metal deficiencies induced by its chelating ability.

Growth factors. Thiamine HCl (0·2 mg./100 ml.), biotin (0·5 μg./100 ml.) and riboflavin (5·0 μg./100 ml.) appear to be absolute requirements. 'Reduced sulphur', i.e. compounds more reduced than sulphates on the biosynthetic pathway to cystine and methionine, is also an absolute requirement (Table 3).

Stimulatory factors. In the presence of either glucose or gluconate and the other required nutrients, acetate (0·04% Na acetate trihydrate) was stimulatory. Cyanocobalamin (vitamin B_{12}) was slightly stimulatory in the presence of L-cystine (4·0 mg./100 ml.). This stimulation was also evident when the cystine was replaced by thiosulphate; DL-methionine (3·0 mg./100 ml.) as the sole source of reduced sulphur yielded the same amount of growth as that permitted by cyanocobalamin *plus* either cystine or thiosulphate. It increased the density of growth from about 0·75 to 0·95. Table 3 combines several experiments indicating the need for reduced sulphur carried out under similar conditions.

Table 3. *Reduced sulphur requirement for* Bacillus circulans *mesophil in gluconate medium*

	Sulphur compounds (mg./100 ml.)	Optical density
None	0	0
L-Cystine	1·0	0·44
	4·0	0·66
	8·0	0·78
DL-Methionine	3·0	0·36
	5·0	0·56
	10·0	0·62
L-Cysteine hydrochloride . H_2O	1·0	0·52
	4·0	0·7
	8·0	0·82
Methionine sulphoxide	3·0	0·22
	5·0	0·46
	10·0	0·56
DL-Homocystine	1·0	0·28
	4·0	0·56
	8·0	0·72
Glutathione	5·0	0·11
	10·0	0·32
	50·0	0·58
Na_2 thiosulphate $5H_2O$	10·0	0·38
	50·0	0·52
	100·0	0·78
Na_2SO_4 (anhydrous)	10·0	0
	50·0	0
	100·0	0

Citric acid cycle components (DL-malate, fumarate, succinate, citrate) tested through a wide concentration range (0·01–0·5), and related compounds (L-glutamate, DL-alanine, and DL-aspartate) neither supported growth in the absence of glucose or gluconate nor were they stimulatory in the presence of glucose or gluconate.

Bacillus circulans—*thermophilic strain*

Substrates. Initial pH 6·0. Glucose, sucrose, gluconate, or glycerol in concentrations of 1·0 %, in combination with (*a*) L-glutamate (0·5%), or (*b*) succinate (0·3%) + L-arginine hydrochloride (0·05%) + histidine hydrochloride (0·02%) supported dense growth (O.D. 1·7). Glutamate or the succinate + arginine + histidine combination supported moderate growth (O.D. 0·7). The addition of acetate (0·04% Na acetate trihydrate) to these media decreased the lag time. The requirement for glutamate or for succinate + arginine + histidine is absolute.

Bacillus licheniformis—*mesophilic strain*

Substrates. Initial pH 6·5. Glycerol (1·0 %) or glucose (1·0 %). Sucrose or gluconate did not support growth.

Growth factors. The following amino-acids represent absolute requirements: DL-alanine (0·05%), DL-aspartate (0·05%), L-glutamate (0·5%), glycine (0·05%) L-arginine hydrochloride (0·05%), L-histidine hydrochloride (0·02%), and DL-lysine hydrochloride (0·05%).

Stimulatory factors. The addition of cytidylic acid or guanylic acid (up to 0·01 %) increased growth from O.D. 0·45 to 0·9; the corresponding nucleosides and free purine and pyrimidine bases were inactive. Alkali-hydrolysed yeast nucleic acid was stimulatory to the expected extent. The nucleic acid was hydrolysed by suspending yeast nucleic acid in water, bringing the preparation to pH 9·5, autoclaving for 5 min. at 115–120°, then neutralizing.

This organism grew in flakes; the others grew diffusely.

Bacillus licheniformis—*thermophilic strain*

Substrates. Initial pH 6·5. Glycerol (0·5 %), glucose (0·5 %) or gluconate (0·05 %) allowed growth in the presence of glutamate or succinate. Glucose or gluconate was not as effective as glycerol. A combination of L-arginine hydrochloride (0·05 %) + L-histidine hydrochloride (0·02 %) + L-proline (0·005 %) also supported growth in the glycerol medium. It was advantageous to add 1·0 mg./100 ml. additional Mn for this strain.

The utilization of intermediates of the citric acid cycle in glycerol media was surveyed. DL-Malate (up to 0·1 %) supported growth. Citrate (as tripotassium citrate monohydrate, 0·05 %) *plus* either Na acetate trihydrate (0·01 %) or Na fumarate (0·1 %) also supported growth. Acetate or fumarate alone, tested up to the toxic limit, did not support growth. Potassium citrate monohydrate was not employed in concentrations above 0·05 % because of the induction of metal deficiencies through its chelating ability.

Sodium bicarbonate added aseptically to the glycerol medium in the absence of citric acid cycle intermediates allowed dense growth (O.D. up to 1·2). When the pH of the medium was raised to 7·0, protogen (supplied as DL-thioctic acid, 6 : 8-dithiooctanoic acid, for which we are indebted to Dr E. L. R. Stokstad of the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N.Y.) at 40 mμg./100 ml. supported growth in the presence of 0·03 % Na acetate trihydrate; higher concentrations of acetate were toxic. Thiamine hydrochloride (0·1 mg./100 ml.) replaced thioctic acid in the presence of acetate under these conditions.

Bacillus stearothermophilus—*facultative thermophilic strain*

Substrates. Initial pH 6·0. Glucose (1·0 %) or sucrose (1·0 %). The addition of 0·04 % acetate as Na acetate trihydrate was stimulatory.

Growth factors. Thiamine hydrochloride (15μg./100 ml.), biotin (0·9μg./100 ml.), and nicotinic acid (0·15 mg./100 ml.) represent absolute requirements.

Stimulatory factors. The addition to the glucose medium of a combination L-arginine hydrochloride (0·01 %) + L-histidine hydrochloride (0·0045 %) + DL-isoleucine (0·04 %) doubled growth (final O.D. 1·0).

DISCUSSION

Most recent attempts to explain thermophily ascribe such growth to an exceptionally rapid synthesis of enzymes which outstrips the thermally accelerated destruction of enzymes. That is to say, thermophily is viewed as an active process and not solely as a result of the possession of thermostable key enzymes (Allen, 1953; Clegg & Jacobs, 1953). This postulated rapidity of synthesis and destruction of cell substance enhances the need for culture media which allow the organism to carry out essential syntheses at the highest possible rates. Such nutritionally adequate media should also, by simple modification, lend themselves to the manipulation of inorganic and organic components. For instance, in studying proteolytic enzymes it might be desirable to measure their production and activity as a function of concentration of the divalent ions active as co-factors. The relatively inexpensive defined media devised appear to be adequate for mass cultures, despite their simplicity; supplementation with complex materials (yeast autolysate, liver extracts, peptones, etc.) seldom increased growth more than 0·2–0·4 O.D. units.

Since this investigation was a survey of growth requirements in so far as this knowledge was needed for mass cultures, neither analysis of the kinetics of any isolated metabolic reaction nor an exhaustive analysis of any of the various nutritional patterns encountered was attempted; at this stage final growth was the index of adequacy of culture media. It proved necessary to inspect cultures at frequent intervals to avoid missing peak growth because rapid autolysis was likely to follow the attainment of full growth (Allen, 1950). These visual inspections of growth gave the impression that growth under these conditions was more rapid in the media which permitted the denser final growths.

Oxygen supply probably most commonly limits growth of these cultures in complete media. At elevated temperatures oxygen consumption, as indicated in the reviews previously cited, is accelerated; concurrently the solubility of oxygen in water is diminished. The use of media in shallow layers was mandatory in the technique employed here.

The diversity of nutritional requirements encountered with aerobic spore-forming bacteria suggests that they are biochemically more heterogeneous than is suggested by their shared morphological features. Even within a single species, e.g. *B. licheniformis*, the heterogeneity is striking. The findings of Campbell & Williams (1953) indicate that a comparable heterogeneity holds for the obligate thermophils. They showed that strains of *B. stearothermophilus* exhibited a variety of growth requirements; in many strains, moreover, these requirements depended on the temperature. In most instances, as in 'temperature' mutants, cultures incubated at the higher temperatures had additional exogenous requirements; less frequently the opposite was true. Consequently, the 'absolute' requirements listed in the present paper may not hold for other than the stated temperatures. The nutrition of the aerobic spore-formers as outlined to date gives the impression of a sampling of a diversity of nutritional patterns.

The only novel phenomenon noted in the present work was with an aberrant agar-liquefying strain of *B. stearothermophilus*—a mutual sparing between riboflavin and calcium. Allen (unpublished) has found this phenomenon to be common among bacilli. The complexity of this calcium-riboflavin relation precludes a detailed treatment at this time. In any event, the media devised to date, on the basis of preliminary experiments with additional strains, appear to be generally suitable for mass cultures. Several strains of facultatively thermophilic *B. stearothermophilus* obtained from the National Canners Association, Washington, D.C., grew to O.D. 2·4 when the basal medium was supplemented with glucose, acetate, and the vitamins and amino-acids found necessary by Campbell & Williams (1953). A thermophilic strain of *B. coagulans*, no. 139, obtained from Dr Allen, grew to O.D. 2·8 in the basal medium supplemented with glutamate plus either glycerol or carbohydrate. A mesophilic *B. sphaericus* (no. RA91, obtained from Dr Allen) reached O.D. 2·2 in the basal medium supplemented with glucose, acetate, and amino-acids.

The nutritional patterns of the five strains discussed here may have their counterparts elsewhere in microbial nutrition. A requirement similar to that for 'reduced sulphur' in the *B. circulans* mesophil was described for certain mutants of *Bacterium coli* (Lampen, Roepke & Jones, 1947), in Saprolegniaceae (Reischer, 1951), and in *Proteus morganii* (Meyers & Porter, 1944), to list only non-parasitic bacteria; it has not hitherto been reported in bacilli. The growth stimulation of the *B. circulans* mesophil by cyanocobalamin or methionine accords with evidence which implicates cyanocobalamin in methylations. This raises the question of whether the methionine requirements reported by Campbell & Williams for several strains of *B. stearothermophilus* represent either (*a*) a requirement for 'reduced sulphur', (*b*) a specific requirement for methionine, or (*c*) a deficiency in the synthesis of labile methyl groups. The 'glutamate' requirement for the *B. circulans* and *B. licheniformis* thermophils resembles the stimulation described by Wiame & Storck (1953) for a mutant of *B. subtilis*; glutamate here was replaceable, as with the *B. licheniformis* thermophil, by proline. The activity of succinate in replacing glutamate for the *B. circulans* thermophil suggests that the 'glutamate' requirement is essentially a requirement for a citric acid cycle component, and that permeability factors and restrictions in transamination underlie many of the differences among strains. To chart the interrelationships governing the utilization of carbohydrate, glycerol, acetate, citric acid cycle intermediates, and the requirements for thiamins, biotin, and pantothenate, is a task as yet attacked only in piecemeal fashion in metazoan and microbial nutrition. A glutamate requirement for a strain of *B. cereus* was mentioned by Foster & Heiligman (1949). The basis for the requirement for carbohydrate in *B. stearothermophilus*, unexpected in an aerobic organism, is obscure. The non-accumulation of acid suggests that this organism derives energy from oxidation as well as glycolysis. Hendlin (1949) described a strain of *B. subtilis* which required carbohydrates; citric acid cycle intermediates did not support growth.

The stimulation by nucleotides of the *B. licheniformis* mesophil and the inactivity of adenosine contrasts with the repeated observation that adenosine is required for spore germination in *Bacillus* (Pulvertaft & Haynes, 1951; Stewart & Halvorsen, 1953).

Interactions among protogen, thiamine, malate, acetate, and bicarbonate such as that detected in the thermophilic *B. licheniformis* may become familiar now that synthetic protogen is available in the form of DL-thioctic acid.

A similar interaction was described by Lytle, Zulick & O'Kane (1951) for *Streptococcus faecalis*; with gluconate as substrate, acetate was interchangeable with protogen; and malate, succinate, or bicarbonate were in turn interchangeable with protogen or acetate. Sparing of thiamine by protogen + acetate was described for a mutant of *Bacterium coli* (Reed & DeBusk, 1952).

Non-metabolizable EDTA replaced the citrate formerly employed as metal buffer and allowed a clearer demonstration of the relations of substrate to other nutrients. As mentioned, *trans*aconitic acid buffer was considered to be unattacked under the present cultural conditions, but there is the possibility that under certain circumstances it may be convertible to the metabolizable *cis* isomer. In future work it is hoped to replace the potentially metabolizable aconitic acid with buffers less likely to be physiologically active.

The studies at the Mount Sinai Hospital were assisted by a grant from the Office of Naval Research, and those at Haskins Laboratories by grants from the Lederle Laboratories Division of the American Cyanamid Co. and the Alfred L. Loomis Foundation.

REFERENCES

Allen, M. B. (1950). The dynamic nature of thermophily. *J. gen. Physiol.* **33**, 205.

Allen, M. B. (1953). *Bact. Rev.* **17**, 125.

Campbell, L. I. & Williams, O. B. (1953). The effect of temperature on the nutritional requirements of facultative and obligate thermophilic bacteria. *J. Bact.* **65**, 141.

Clegg, L. F. L. & Jacobs, S. E. (1953). Environmental and other aspects of adaptation in thermophiles. *Symp. Soc. gen. Microbiol.* **3**, Adaptation in microorganisms.

Crook, E. M. (1951). Specificity and mode of action of proteolytic enzymes. *J. Soc. Leath. Tr. Chem.* **35**, 257.

Foster, J. W. & Heiligman, F. (1949). Biochemical factors influencing sporulation by a strain of *Bacillus subtilis*. *J. Bact.* **57**, 639.

Hendlin, D. (1949). The nutritional requirements of a bacitracin-producing strain of *Bacillus subtilis*. *Arch. Biochem.* **24**, 435.

Hutner, S. H. (1950). Anaerobic and aerobic growth of purple bacteria (Athiorhodaceae) in chemically-defined media. *J. gen. Microbiol.* **4**, 286.

Hutner, S. H., Provasoli, L. & Filfus, J. (1953). Nutrition of some phagotrophic fresh-water chrysomonads. *Ann. N.Y. Acad. Sci.* **55**, 852.

Knight, B. C. J. G. & Proom, H. (1950). A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. *J. gen. Microbiol.* **4**, 508.

Lampen, J. O., Roepke, R. R. & Jones, M. J. (1947). III. Mutant strains of *Escherichia coli* unable to utilize sulfate for their complete sulfur requirements. *Arch. Biochem.* **13**, 55.

Lytle, V. L., Zulick, S. M. & O'Kane, D. J. (1951). Replacement of the pyruvate oxidation factor by carbon dioxide. *J. biol. Chem.* **189**, 551.

Meyers, F. P. & Porter, J. R. (1944). The nutrition of *Proteus morganii*. Sulphur requirements. *J. Bact.* **47**, 29.

Pulvertaft, R. J. V. & Haynes, J. A. (1951). Adenosine and spore germination; phase-constrast studies. *J. gen. Microbiol.* **5**, 657.

Reed, L. J. & DeBusk, B. G. (1952). A conjugate of α-lipoic acid required for oxidation of pyruvate and α-ketoglutarate by an *Escherichia coli* mutant. *J. biol. Chem.* **199**, 873.

Reischer, H. S. (1951). Growth of Saprolegniaceae in synthetic media. I. Inorganic nutrition. *Mycologia*, **43**, 142.

Smith, E. L. (1951). Proteolytic enzymes. In *The Enzymes.* I, pt. 2, p. 793. Edited by Sumner, J. B. and Myrbäck, K., New York: Academic Press.

Stewart, B. T. & Halvorsen, H. O. (1953). Studies on the spores of aerobic bacteria. I. The occurrence of alanine racemase. *J. Bact.* **65**, 160.

Wiame, J. M. & Storck, R. (1953). Métabolisme de l'acide glutamique chez *Bacillus subtilis. Biochim. biophys. Acta*, **10**, 268.

(*Received* 12 *June* 1953)

Lindegren, C. C. & Pittman, D. D. (1953). *J. gen. Microbiol.* 9, 494–511.

The Induction in a *Saccharomyces* sp. of the Gene-mutation controlling Utilization of Galactose by Exposure to Galactose

By C. C. LINDEGREN and D. D. PITTMAN

Biological Research Laboratory, Southern Illinois University, Carbondale, Illinois, U.S.A.

SUMMARY: The Carbondale yeast stock contains cultures which are characterized by the delayed fermentation of galactose. Long-term adaptation to galactose results from gene-mutation followed by the selection of the mutant in the presence of galactose. The original non-fermenter and the derived fermenter mutant contain no galactozymase and possess no selective advantage over each other when grown in glucose. Three different methods (variance analysis, fluctuation-redistribution tests, and chemostat experiments) which have been used with success in the detection of spontaneous mutations showed that the mutation to ability to ferment galactose did not occur spontaneously in these stocks. Exposure of non-dividing non-fermenter cells of the long-term fermenter to galactose induced the mutation to the fermenter type in a small but relatively constant number of cells, while exposure to glucose, ribose or lactose was ineffective; dividing cells were not susceptible to the mutagenic action of galactose. Prolonging the exposure of the cells on galactose agar plates increased the number of mutations. It is inferred that the induction of the mutation results from the impingement of galactose on the gene-surface thus modelling the gene-surface into a mirror-image of the substrate.

The phenomenon of long-term adaptation to galactose in the Carbondale yeast stock was shown by Mundkur & Lindegren (1949) to result from gene-mutation, followed by selection of the mutant in the presence of galactose. Tetrad analysis of hybrids between haploid galactose-positive mutants originating from the long-term adapted cultures and galactose-negative cultures carrying several other markers proved that each rapid-fermenter culture possessed a gene for galactose fermentation which segregated regularly in the hybrid and, therefore, that gene mutation had occurred. Mundkur (1952) used the Luria-Delbrück (1943) method for distinguishing between spontaneous and induced mutations and found the variance approximately equal to the mean. He showed that the phenomenon of long-term adaptation in the Carbondale stocks is specifically different from long-term adaptation occurring in *Saccharomyces chevalieri* analysed by Winge & Roberts (1948), and that the generalizations of Spiegelman and his collaborators (1950, 1951) concerning *S. chevalieri* do not apply to the long-term fermenters in the Carbondale stocks.

It has been shown recently that the non-fermenters of the Carbondale stock are incapable of producing galactozymase. A series of experiments has revealed that many misconceptions concerning the phenomenon of the adaptation of rapid fermenters of galactose arose from the occurrence of impurities in the galactose (Lindegren & Palleroni, 1952; Sheffner & Lindegren, 1952). Tests with pure galactose revealed that rapid fermenters of galactose are incapable of adapting to the utilization of galactose unless supplied with some other source of energy. The non-fermenters of the Carbondale stock are incapable of fermenting pure galactose, contain no detectable galactozymase, and do not (as Lindegren once proposed, 1949) possess a subthreshhold capacity for galactozymase production, too small to be registered by the Durham tube test. These non-fermenters are the result of an intensive genetical selection which has produced a paired stock: one member incapable of fermentation, the other capable of vigorous fermentation (Lindegren, 1953).

Long-term adaptation in the Carbondale stocks involves two steps: (*a*) the mutation from the non-fermenter gene to the dominant allele, (*b*) the adaptation of the derived fermenter to galactose fermentation through the utilization of some source of energy other than galactose. In Mundkur's experiments these two phenomena were inextricably joined. This paper reports a series of experiments with the objective of separating the two events. They reveal that (*a*) the mutation cannot be demonstrated to occur spontaneously, but (*b*) the mutation can be induced by exposure to galactose but *not* by exposure to glucose, ribose or lactose (the latter two substrates are not utilizable by the cell).

METHODS AND MATERIALS

Cultures. Culture 5991 (*Saccharomyces* sp.) of the Carbondale stock is a long-term fermenter of galactose, closely related to culture 6166 analysed by Mundkur. When a number of different transfers from this culture are tested simultaneously in Durham tubes containing full nutrient medium and galactose, fermentation occurs at different times after prolonged delays. The variant cells from a positive tube on transfer to galactose medium cause fermentation to occur within 24 hr. In the present study, three rapid fermenters of galactose were derived from 5991 by plating cells on galactose plates and selecting three of the large colonies. Each is inferred to carry the mutant gene which controls the rapid fermentation of galactose. Since the fermentation of galactose is an adaptive process, the fermenter is enzymically similar to the non-fermenter when grown in glucose, for in 'short-term' adaptation the enzyme appears in the cells of fermenters only after from 2–3 hr. exposure to galactose. Neither the non-fermenter nor the fermenter contain enzyme during growth on glucose.

Culture 5991 is a fermenter of maltose, melibiose and sucrose, and does not grow in the absence of adenine, thiamine, pyridoxine or inositol. It is capable of utilizing lactate, acetate, pyruvate and formate.

Media

Purified galactose. Galactose was purified by treating a solution with yeast to metabolize the glucose and drying the resultant solution *in vacuo* to crystals. The crystals were dissolved in water and evaporated to a thick syrup which was refrigerated as the purified stock reagent.

Complete nutrient media. The complete nutrient medium contained: peptone, 3·5 g.; liquid yeast extract, 4 ml.; $(NH_4)_2SO_4$, 4 g.; $MgSO_4$, 1 g.; KH_2PO_4, 2 g.; glucose, 20 g.; water, 1 l. This medium was made into galactose complete agar by adding 22 g. Difco agar and substituting galactose for glucose.

Eosin methylene blue purified galactose agar. (EMB-PG) agar was prepared by adding to 1 l. of water the following materials: peptone, 3·5 g.; $(NH_4)_2SO_4$, 2 g.; $MgSO_4$, 1 g.; KH_2PO_4, 2 g.; Difco yeast extract, 2·5 g.; purified galactose, 20 g.; agar, 22 g.; eosin solution (2 %), 20 ml.; methylene blue solution (0·5 %), 5 ml. This medium gives the same results as that used by Spiegelman, Sussman & Pinska (1950), producing the characteristic colour reactions of non-fermenter and fermenter clones.

Chemically-defined media. Defined glucose medium was prepared by dissolving in 1 l. of water the following constituents: glucose, 20 g.; KH_2PO_4, 1·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $CaCl_2.2H_2O$, 0·33 g.; $(NH_4)_2SO_4$, 4 g.; KI, $2{\cdot}5 \times 10^{-5}$ g.; the metals B, Cu, Mo, Mn, 0·0025 p.p.m. each; Fe, 0·0013 p.p.m.; Zn, 0·0175 p.p.m. Thiamine, pyridoxine, niacin, pantothenate, 200 μg.; biotin, 2 μg.; inositol, 0·01 g.; adenine and methionine 2 mg. each. Defined galactose medium was prepared by the substitution of purified galactose for glucose in the above medium.

'*Non-nutrient*' *agar.* Agar medium designated as 'non-nutrient' contained 22 g. Difco agar/l. water. This medium was used to cover galactose nutrient agar; by itself it did not support any visible growth.

Experimental procedure

Variance analysis. In all the analyses reported here, non-fermenter cells of culture 5991 were grown from 24 to 48 hr. at 30° in full nutrient glucose broth in Erlenmeyer flasks on a shaker. A small sample of broth was centrifuged, the supernatant fluid discarded and the cells serially diluted in M/15-potassium dihydrogen phosphate to a concentration of less than 1000 cells/ml. Twenty-five to thirty 'growth tubes', containing 2 ml. each of glucose-defined medium, were inoculated with 0·1 ml. of diluted culture. Direct plate counts on glucose agar were made with at least two of the 'growth tubes' in each series to determine the number of cells in the inoculum. The 'growth tubes' were incubated for from 36 to 48 hr. and replicate haemacytometer counts were made on at least three tubes to determine the final population. The supernatant fluid was discarded after centrifugation, the cells resuspended in M/15-potassium dihydrogen phosphate, and the suspension used to inoculate galactose plates.

The inoculum was sufficiently small to ensure detection of fermenter mutants carried in it and to exclude the chance of seeding a population in equilibrium with respect to fermenters and non-fermenters. Centrifugation and dilution served to eliminate much of the original nutrient and to concentrate the cells previous to plating small volumes on EMB–PG agar. Pour plates were made by circulating molten galactose agar (39–43°) in Petri dishes containing equal sample volumes of washed cell suspensions. To prevent growth on the agar surface and to impose anaerobic conditions, each pour plate was layered with approximately 12 ml. of 'non-nutrient' agar. In experiments employing EMB–PG agar, plates were rotated and cells spread evenly over the surface with a sterile, bent glass rod.

Stability of the derived mutant. The stability of the rapid fermenters of galactose derived from non-fermenter culture 5991 was investigated, since any considerable amount of reverse mutation could have serious effects on the analysis. A derived mutant which had been subcultured on glucose for more than 12 months yielded large galactose fermenter colonies on galactose plates and fermented galactose in fermentation tubes in from 18 to 24 hr. Mass reversion to inability to ferment galactose as a result of ultraviolet irradiation has not been detected in this culture. In this respect, it was quite unlike the galactose-fermenting yeast described by James (1952) in which as many as 10% of the surviving cells lost the ability to ferment galactose following irradiation. Fermenter cells from the derived culture were irradiated to 3% survival and plated on EMB–PG agar. The plates were covered with the typical red colonies characteristic of the rapid fermenter genotype; no pink colonies were detected. Plating irradiated cells on glucose agar before testing by Durham fermentation tubes gave similar results. The possibility that the non-fermenter cells produced by irradiation might have been incapable of surviving direct transfer to EMB–PG is not necessarily excluded, but the results are apparently different from those found by James.

RESULTS

Preliminary experiments

Growth of non-fermenters on galactose plates. The number of mutants appearing on galactose complete medium is not directly proportional to the number of cells seeded on the plate, although it may bear a relation to the final titre of cells on a plate. A direct microscopic examination of inoculated plates revealed that non-fermenter cells go through one to six divisions during the first 24 hr. after which the growth of clumps and single cells is greatly retarded. Attempts to prepare a medium which is completely incapable of supporting growth of non-fermenters but capable of supporting the growth of mutant fermenters were unsuccessful; even on defined medium with purified galactose from one to five divisions occurred, probably due to the presence of endogenous reserves and the failure to eliminate completely the nutrient after centrifuging the inoculum. Variation of the inoculum from 10^5 to 10^8 cells/plate gave no significant difference between the total number of mutants per plate.

Absence of competition between non-fermenter and mutant fermenter in glucose medium. A series of test-tubes containing 2 ml. of defined medium with glucose as sole added carbohydrate were inoculated with a mixed population consisting of approximately 250,000 non-fermenter and 350 fermenter cells. The fermenter cells were obtained from a 48 hr. complete nutrient glucose broth culture inoculated with cells from a rapid fermenter originally derived from culture 5991, but which had been carried for 12 months on glucose slopes. The tubes were incubated at 30° on a reciprocal shaker and were sampled for total populations of fermenters and non-fermenters by the plate count technique (Fig. 1*a*). Samples were plated on galactose agar and glucose agar plates. The total count of the cells was obtained by colony counts on glucose agar;

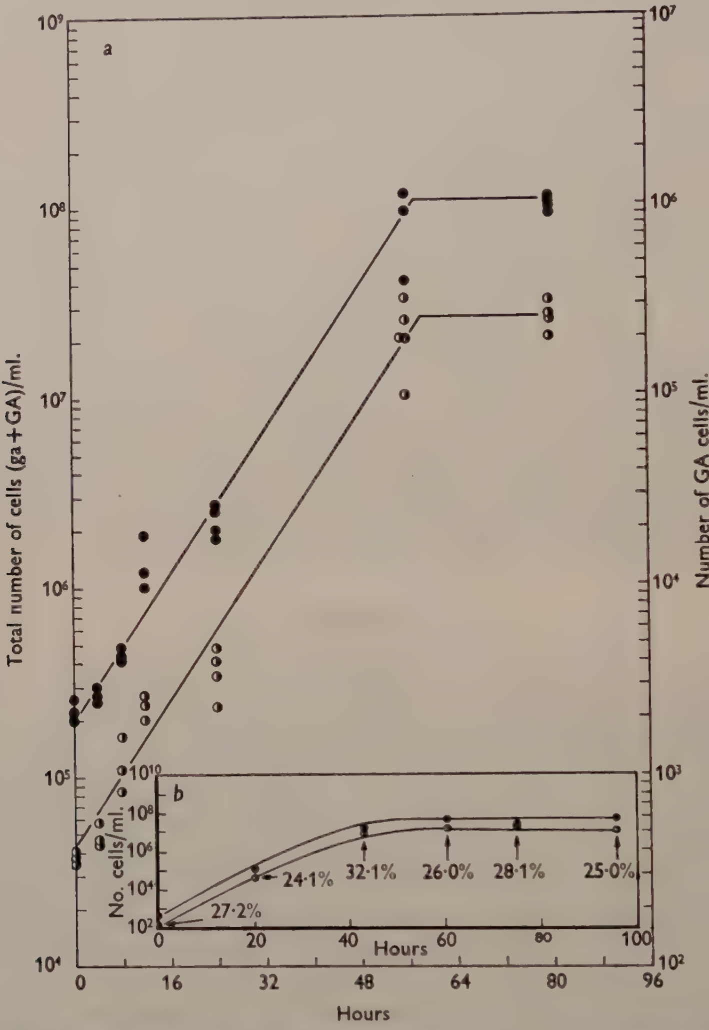


Fig. 1. Population analyses of mixed cultures of fermenter (GA) and non-fermenter (ga) cells. Fig. 1*a* presents the results of growing populations in defined glucose medium; Fig. 1*b* depicts the growth curves of a mixed population grown on complete glucose medium. The parallel growth curves under both conditions indicate the absence of selective factors favouring either type.

counts on galactose agar gave the numbers of galactose fermenters. Three serial dilutions were made of each sample; each dilution was plated and read after incubation for 3 days. The numbers of galactose fermenter colonies appearing on the plates were proportional to the dilutions, showing that the growth curves (Fig. 1) are accurate reflexions of the population sampled and not in error due to growth of non-fermenters on the plates. The difference between the two values gave the number of non-fermenters. The parallel growth curves of fermenter and non-fermenter populations indicate that the non-fermenter has no selective advantage over the fermenter and establish the absence of mass reversion of fermenter to non-fermenter.

A chemostat (Novick & Szilard, 1950) was set up containing complete medium with glucose as the carbohydrate source, and inoculated with *c.* 534 cells of the original non-fermenter culture 5991 and *c.* 200 cells of a galactose fermenter mutant derived from 5991. The experiment ran over 5 days (developing a population density of $6{\cdot}9 \times 10^7$ cells/ml.). The parallel growth curves (Fig. 1*b*) indicate the absence of a selective advantage of either type over the other.

Variance analysis

The experiments described in Table 1 were undertaken for the purpose of detecting variations in the numbers of colonies appearing on galactose when pour plates were inoculated with samples from a single culture. In each experiment ten pour plates were made with 1 ml. of cells from a single tube. The readings were made on the fifth day, and the numbers of mutants appearing on the plates were recorded. The statistical analysis reveals that no significant fluctuation was encountered as a result of sampling error ($P=0{\cdot}2$–$0{\cdot}9$).

Table 1. *The numbers of fermenter mutants detected on replicate platings from the same culture*

	No. of mutants detected			
Sample	Exp. 11	Exp. 18*a*	Exp. 18	Exp. 10*a*
1	15	6	9	10
2	9	11	11	8
3	10	16	9	14
4	8	12	11	12
5	10	15	8	16
6	18	10	10	12
7	5	8	9	13
8	12	15	5	14
9	8	9	8	19
10	9	11	10	15
Average	10·4	12·5	9	13·3
Variance	14·0	12·4	3·1	9·5
χ^2	12·13	8·9	3·1	6·4
P	0·2	0·4	0·9	0·3

The data from a series of variance analyses following the procedure already described are given in Table 2. Exps. A and B present the results of fluctuation tests in which cells were plated on agar containing synthetic medium and purified galactose. The variance closely approximates the mean, but the average number of mutants/plate/day is slightly lower on defined medium than on complete medium (Exps. C–E, Table 2). This may be because yeasts generally adapt and grow slowly in defined medium.

Since as many as five divisions may occur when non-fermenter cells are plated on galactose agar (or on nutrient agar containing no added carbohydrate), the mutants detected after incubation could be the result of mutations occurring during growth on glucose in the culture tube before plating the population on galactose and during residual growth on the agar plates after contact with galactose. If mutations do occur at both sites, magnifying the number of mutant cells present at either locality should, therefore, produce results differing from those obtained in routine variance analyses. In Exps. C and D an opportunity was afforded for the growth and selection of any fermenter mutants present in the inoculum. Glucose-grown non-fermenter cultures were centrifuged, the supernatant discarded, and the cells resuspended in M/15-potassium dihydrogen phosphate with 0·1 ml. of 4 % galactose broth. The cultures were incubated for 7 hr., after which pour plates were made on galactose agar. By the aid of tables supplied by Lea & Coulson (1949), the mean number of mutations/culture was calculated to be 3·6 with a P of 0·224 that a single culture would be detected containing between nine and sixteen mutants. In considering the selective environment and the clumping nature of culture 5991 during rapid growth, an upper range of from twenty to eighty mutants would have been expected in some cultures. No large fluctuations in the numbers of mutants were found, however, after passing the cultures through the selective medium.

Cultures in routine fluctuation tests are derived from small inocula to decrease the chance of seeding mutants in the growth tubes. When a given mutation occurs with a certain frequency (e.g. 1×10^{-8}), it is unlikely that it will occur during the first few divisions of a small population. A large inoculum, however, should lead to large numbers of mutants since a large inoculum is more likely (*a*) to transfer randomly-distributed mutant cells which could multiply during the early divisions, and (*b*) to give rise to mutant cells during the first few divisions. With this in mind, fluctuation tests were made when using large inocula. The results in Exps. E and F (Table 2) show that the mean number of mutants/plate is low and essentially the same as for cultures derived from small inocula. The plates in Exp. F were scored after 6 days. The higher mean is not due to the delayed expression of mutants originally present, but due to prolonged incubation of the plates. This is proved by comparison with results of Exp. G in which a small inoculum of fifty cells was used and the plates were scored after 6 days incubation.

Fluctuation-redistribution experiments

Newcombe (1949) developed a modified fluctuation test which exploits the fact that mutant cells form colonies on solid medium. The cells in the colonies are redistributed over the agar after varied periods of incubation. He found that when mutation to virus resistance occurred before redistribution and exposure to phage, redistribution of the cells after incubation magnified the number of mutants by dispersing the cells of mutant clones.

When mutation to the form able to ferment galactose occurs before plating cultures, rapid fermenter variants which arose before sampling would be selected on the galactose plates and a larger number of fermenter mutant colonies would appear on the plates in which the clones had been redistributed.

Table 2. *The number of fermenter mutants detected after plating a series of similar cultures on galactose agar plates*

Exp. ...	A	B	C*	D*	E	F	G
No. of cultures ...	25	25	25	25	25	25	25
Vol. of cultures/ml. ...	5	5	2·1	2·1	2·1	2·1	2·1
Vol. of samples/ml. ...	1	1	1·0	1·0	1·0	1·0	1·0
Inoculum ...	50	50	15	15	1×10^6	1×10^6	50
Days of incubation ...	5	5	4	4	4	6	6
Culture no.	No. of fermenter mutants						
1	8	15	8	7	10	19	28
2	5	7	6	9	7	18	18
3	5	5	12	6	7	12	14
4	4	10	9	9	10	17	12
5	7	9	12	7	5	16	12
6	6	5	8	13	7	19	14
7	4	3	12	6	11	21	19
8	6	7	7	10	10	19	19
9	5	8	10	15	8	18	20
10	7	15	12	10	6	20	25
11	4	4	15	13	7	12	20
12	6	4	8	9	6	21	24
13	7	7	14	9	10	14	21
14	8	12	8	13	11	20	23
15	7	6	14	12	7	14	24
16	4	4	15	9	9	17	23
17	6	5	11	9	11	15	14
18	10	8	9	8	8	19	21
19	5	5	11	11	7	18	13
20	15	5	10	13	4	16	12
21	12	6	6	13	13	15	18
22	11	6	9	10	8	15	15
23	3	9	11	6	12	18	21
24	5	6	7	8	5	21	12
25	7	7	10	10	13	20	21
No. cell/ml. $\times 10^7$	3·2	3·0	6·7	6·6	24	18	5·8
Average	6·68	7·12	10·16	10·12	8·48	17·4	18·52
Variance	7·89	9·94	7·06	9·11	6·34	7·24	19·68
χ^2	28·36	33·52	16·67	21·60	17·95	9·98	25·50
P	0·3	0·1	0·7	0·6	0·81	0·9	0·4

* Cultures passed through a selective medium by adding 0·1 ml. 4% galactose broth to washed cells suspended in 1 ml. of M/15-KH_2PO_4.

When mutation does not occur before plating, no significant difference is to be expected between redistributed ('R') and undisturbed ('U') plates. A series of tests (shown in table on p. 502) revealed that when the cells of deliberate mixtures were redistributed after short periods of incubation striking increases did occur in the numbers of fermenter colonies.

Duplicate EMB-PG agar plates were paired, and both members of each pair were inoculated with cells from the same tube. In all analyses cultures were first centrifuged, the supernatant poured off and the cells resuspended in half the original volume of M/15-KH_2PO_4. One-tenth ml. samples from each tube were spread evenly on the plates. The plates were incubated for limited periods of time after which 0·1 ml. of sterile phosphate buffer was added to one plate (marked 'R') of each pair and the cells redistributed by spreading for approximately 30 sec.

Fermenter inoculum (no. of cells)	Fermenter colonies; Redistribution after			Inoculum mixed with 10^6–10^7 Non-fermenter cells/plate		
	2 hr.	6 hr.	10 hr.	2 hr.	6 hr.	10 hr.
	No. of colonies					
495	510	535	> 1000	426	711	> 1000
566	518	673	> 1000	486	598	> 1000
49	57	87	138	74	83	151
40	39	56	195	38	120	175
201		801			870	
104		1112			1170	

The length of the incubation period elapsing before redistribution should be sufficiently long to insure that a detectable increase in mutant colonies will result from the multiplication of mutant fermenters on the plate. Control experiments showed that a minimum of 6 hr. was sufficient to produce a significant difference between redistributed and undisturbed plates. The incubation period should not be so prolonged that the effect of the substrate, if any, might obscure the results. Since rapid fermenter variants become barely visible after approximately 3 days, the incubation time was set arbitrarily at a maximum of 10 hr.

The data shown in Table 3 reveal no significant difference between the average number of mutants detected on plates on which the cells had been redistributed or left undisturbed after incubation periods of 7 or 10 hr. In some pairs the redistributed plate showed a higher number of mutants than the undistributed control. In other pairs the number of mutants which appeared on the redistributed plates was smaller. It may be inferred that the frequency of mutation before redistribution is so small as to be undetectable, and hence that the mutations must have occurred on the plates. The fact that incubation periods of from 7 to 10 hr. did not change significantly the numbers of mutants strongly suggests that mutation did not occur until cell division had ceased or the rate had slowed down considerably. A comparison of Exps. 32, 38 and 41 (Table 3) reveals that the number of fermenter mutant colonies appearing on the galactose agar plates is relatively independent of the number of cells used to inoculate the plate. In these three experiments 3·9, 4·5 and $8{\cdot}8 \times 10^6$ cells were used respectively in each inoculation. No significant difference in the number of fermenter mutants occurred in the 150 cultures analyzed. The counts on these plates were made on the fifth day. Exp. 43 (Table 3) is a parallel experiment to 41, with the exception that the mutants were counted on the thirteenth day. The number of mutant colonies which appeared on the plates in Exp. 43 was almost double the number appearing on the plates counted on the fifth day, thus showing that the number of mutants increased as incubation progressed. The experiment on redistributing the cells shows that no mutants were present in the cells used as an inoculum, since, if that had been the case, the numbers of colonies in some of the redistributed plates should have been larger than the numbers on the undisturbed plates. It may be inferred, therefore, that the mutations occur only on the plates. This view is further supported by the increase in the number of mutants with continued incubation as revealed by the higher counts observed after 13 days.

Table 3. *Numbers of galactose fermenter mutants appearing on paired plates (undisturbed 'U' and redistributed 'R') in a series of fluctuation-redistribution tests (Newcombe,* 1952)

Exp. ...	32		38		41		43	
No. of cultures ...	25		25		25		25	
Vol. of cultures (ml.) ...	2·1		2·1		2·1		2·1	
Inoculum ...	50		100		50		50	
No. of samples ...	0·1		0·1		0·1		0·1	
No. of cells/plate $\times 10^6$ ...	3·9		4·5		8·8		8·8	
Incubation time/hr. ...	7		7		10		10	
	U	R	U	R	U	R	U	R
Culture no.	No. of fermenter mutants							
1	11	10	18	19	15	13	40	49
2	10	13	17	24	16	15	36	27
3	13	19	23	21	21	19	33	41
4	15	14	20	22	19	22	39	37
5	14	9	16	8	10	15	37	33
6	12	10	11	20	12	19	32	35
7	10	12	17	10	15	14	35	32
8	21	12	15	20	13	10	34	40
9	10	14	12	21	17	14	39	36
10	9	13	24	23	13	16	30	41
11	12	11	17	20	10	11	27	28
12	16	20	22	10	12	13	21	25
13	12	13	20	19	25	24	44	34
14	10	13	13	21	18	9	46	29
15	14	19	24	18	13	16	35	32
16	10	12	20	21	17	17	36	21
17	15	13	10	16	8	19	22	32
18	22	16	21	17	18	19	39	41
19	15	16	14	12	12	17	29	37
20	14	14	17	24	25	14	26	36
21	10	11	24	15	11	14	29	40
22	13	9	22	13	15	12	26	35
23	19	10	12	17	17	16	33	31
24	11	9	16	22	11	23	32	34
25	16	15	14	21	19	16	40	45
Average	13·3	13·1	17·5	18·2	15·3	15·9	34·6	34·8
Variance	12·2	9·0	18·5	20·4	19·5	14·6	39·5	39·9
χ^2	22·0	16·5	25·4	26·9	30·6	22·0	27·4	27·5
P	0·5	0·8	0·4	0·3	0·1	0·5	0·2	0·2

The mutability of non-fermenter cells grown under different conditions

The data presented above indicate that mutation from non-fermenter to fermenter does not occur during growth in glucose broth. Table 4 presents several series of variance analyses on culture 5991 in which the cells comprising the inoculum for the galactose pour plates were grown either on media of different composition or under different conditions. Routinely, the growth tubes used to inoculate the galactose plates contained glucose-defined medium; this medium was used in Exp. 45 (Table 4). In Exp. 46 (Table 4) glucose complete medium was used. A parallel pair of experiments in which fructose replaced glucose was also performed. In all four experiments the cultures contained substantially the same number of cells and no significant differences were found between the numbers of mutants appearing on the plates, suggesting that these variations in the medium did not affect the mutability of the cells.

In Exp. 49*a* (Table 4), twenty EMB-PG plates were inoculated with 2×10^6 non-fermenter cells and the number of mutant colonies counted after 5 days. In Exp. 49*b* (Table 4) the inoculum was grown on an EMB plate containing no added carbohydrate, and after 6 days incubation, the cells were washed from the surface of each EMB plate and transferred to molten galactose defined agar. During the 6 days on the EMB plate, the population had increased from 2×10^6 to $3{\cdot}1 \times 10^8$. Washing removes some of the dye, thus giving the galactose plate a distinct pink tint. The fermenter colonies were also faintly coloured. Comparison of Exps. 49*a* and 49*b* shows little difference in the numbers of mutants on the EMB plates containing galactose (49*a*) and on the galactose plates (49*b*) inoculated with $3{\cdot}1 \times 10^8$ cells which had been washed from the EMB plates lacking added carbohydrate.

In Exp. 50 (Table 4) glucose was added to the EMB plate, and the population of cells increased to $6{\cdot}1 \times 10^8$. Plating the cells on galactose agar yielded approximately the same number of mutants as in the two preceding experiments, indicating that neither exposure to EMB nor any of the other conditions imposed by growth on these plates had increased the number of mutants.

Conditions controlling the variation in the numbers of mutants which appear on galactose plates

Luria & Delbrück (1943) in analysing the frequency of mutation of *Bacterium coli* from bacteriophage-sensitivity to bacteriophage-resistance, found that the distribution of bacteriophage-resistant mutants on different plates was characterized by an extremely high variance when plates flooded with bacteriophage were smeared with cells grown in different tubes. They concluded that the mutation occurred spontaneously in the absence of the bacteriophage; if the mutation occurred early in the growth tube, many mutants would be found, but if it occurred late only a few would appear on the plate. The extremely low variance of the distribution of galactose fermenters in experiments carried out according to the Luria-Delbrück procedure raises the question of whether the experiment can be carried out under conditions in which a high variance would be observed.

Non-fermenter cells of culture 5991 were inoculated into full nutrient medium and aerated for 24 hr. The cells were washed, resuspended in phosphate buffer and 1 ml. was added to each tube of two series: (*a*) thirteen tubes containing 6 ml. of 2% glucose in phosphate buffer, and (*b*) eighteen tubes containing 2% reagent galactose in phosphate buffer. The tubes were held for

Table 4. *The numbers of rapid fermenter colonies detected on galactose agar containing non-fermenter populations grown on different substrates*

Exp. ...	45	46	47	48	49*a*	49*b*	50
Inoculum ...	100	100	100	100	2×10^6	2×10^6	2×10^6
Vol. of cultures (ml.) ...	2	2	2	2	0·1	0·1	0·1
Vol. of samples (ml.) ...	1	1	1	1	1·0	1·0	1·0
No. of cultures ...	20	20	20	20	20 (rep)	20 (rep)	20 (rep)
No. of cells/plate ...	58×10^7	$6{\cdot}4 \times 10^7$	$3{\cdot}5 \times 10^7$	$5{\cdot}6 \times 10^7$		$3{\cdot}1 \times 10^8$ (*b*)	$6{\cdot}0 \times 10^8$ (*a*)
Substrate (carbohydrate) ...	Glucose defined medium	Glucose complete medium	Fructose defined medium	Fructose complete medium	EMB (+Gal.)	EMB (−Gal.) (−Glu.)	EMB (−Gal.) (+Glu.)
	No. of mutant colonies						
Culture no.							
1	16	22	28	14	20	17	13
2	15	16	15	19	13	23	14
3	32	23	16	25	23	28	24
4	19	25	21	27	26	24	19
5	17	21	22	15	17	41	20
6	13	20	12	22	9	25	20
7	23	26	22	12	22	29	27
8	18	19	18	19	24	31	29
9	16	34	26	28	12	26	21
10	15	16	19	22	11	21	13
11	27	19	17	19	12	24	12
12	14	15	17	21	16	22	17
13	25	27	22	9	12	22	20
14	13	16	20	19	13	24	12
15	22	18	18	17	18	28	16
16	24	22	15	22	10	23	22
17	22	28	10	27	11	29	20
18	24	27	17	18	20	18	19
19	17	18	22	16	25	32	23
20	17	26	13	28	17	18	19
Average	19·45	21·90	18·50	19·95	16·55	25·25	19·00
Variance	26·78	24·66	20·36	28·51	29·62	31·46	22·65
χ^2	26·16	21·39	20·91	27·1	34·01	23·67	22·63
P	0·12	0·3	0·3	0·1	0·03	0·2	0·2

a, cells suspended in 0·2 % glucose complete broth before spreading on galactose agar plates. *b*, populations determined by assays of four different plates.

4 days at 30°, after which 1 ml. of water containing 2·5 mg. peptone and 0·1 ml. of liquid yeast extract was added to each tube. The population densities after 24 hr. incubation at 30° were found to be $2{\cdot}4 \times 10^6$ in the glucose series and $9{\cdot}6 \times 10^5$ in the galactose series. The complete culture from each tube was mixed with molten galactose agar in a Petri dish and the plates were incubated for 4 days at 30°. The following tabulation shows the number of fermenter colonies appearing on the plates:

Colonies per plate (4 days)	No. of plates	
	Glucose series	Galactose series
1–6	12	2
7–12	1	1
13–18	.	7
19–24	.	6
25–30	.	1
31–36	.	.
37–42	.	.
43–48	.	.
49–54	.	1

The glucose series shows the characteristic low variance which indicates that the mutations only occurred after removal from the glucose broth. The variance of the galactose series is in excess of that expected on random sampling of a homogeneous group, indicating that some mutations occurred in the growth tubes.

Absence of spontaneous mutation of non-fermenter to fermenter in glucose

If spontaneous mutation occurs in the chemostat, the number of mutants detectable on plating should increase linearly with time provided (*a*) that the mutant type is stable, (*b*) that the number of mutants forms a very small fraction of the total population, (*c*) that neither mutant nor non-mutant possesses a selective advantage. If the growth of the mutant type were suppressed by the parent strain, an initial rise in the number of mutant colonies would be followed by levelling off.

Experiments were performed with the chemostat in which populations of non-fermenter cells (Fig. 2) were grown over long periods of time under conditions which simulated those occurring (*a*) in growth tubes containing defined medium, (*b*) in growth tubes containing complete nutrient medium, and (*c*) on agar plates, where the rate of multiplication is greatly reduced after the first few divisions. Non-fermenter cells of culture 5991 were grown for 14 days at a generation time of 12 hr. (30°) on a defined medium with a low concentration of orthophosphate (10 mg. PO_4(L)) at a population density of $2{\cdot}7 \times 10^7$ cells/ml. Samples taken at regular intervals were centrifuged, the supernatant discarded and the cells resuspended to the original volume in sterile vials which were shaken by hand for at least 1 min. before sampling. Since population analyses (Fig. 1*a*, *b*) showed neither suppressive effects nor selective advantages for either fermenter or non-fermenter, it was concluded that the curve (Fig. 2) which represents the numbers of colonies appearing on the galactose plates resulted from mutations occurring on the plates rather than mutation, followed by selection, in the chemostat, although the latter could yield similar results (Novick & Szilard, 1950).

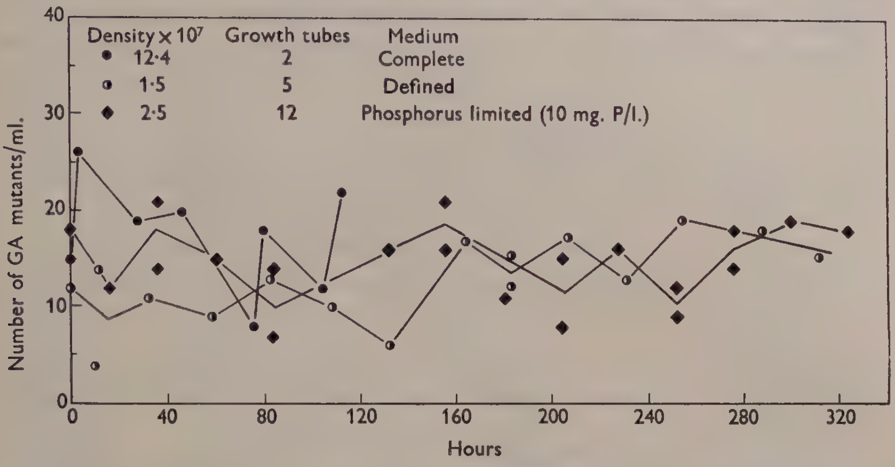


Fig. 2. Experiments with the chemostat in which populations of non-fermenter cells were grown over long periods of time under conditions simulating those occurring: (*a*) in growth tubes containing defined medium, (*b*) in growth tubes containing complete nutrient medium, and (*c*) on agar plates, where the rate of multiplication is greatly diminished after the first few divisions. The results show no detectable increase in GA mutants over periods of continuous growth extending as long as 14 days.

The absence of a detectable spontaneous mutation rate to ability to ferment galactose could be ascribed to a two-step mutation resulting in a deficiency capable of preventing the expression of the mutant type unless a specific nutrient were supplied. Although the growth studies on defined medium (Fig. 1) strongly suggest that this does not occur, non-fermenter cells of culture 5991 were grown for long periods in the chemostat on complete medium and on defined medium. The results (Fig. 2) revealed no significant differences in the numbers of mutant colonies detected on galactose agar. These findings are in agreement with those obtained from variance and redistribution analyses, which show that the mutation to galactose fermentation does not occur while the cells are growing in glucose medium, and that the constant low numbers of mutants which appear on the galactose plates are not due to suppression of fermenters by non-fermenters.

Pretreatment of non-fermenter cells with galactose

Non-fermenter cells from culture 5991 were exposed to reagent galactose (either Merck or Pfanstiehl, without apparent difference) in phosphate buffer, and to phosphate buffer alone, in the absence of an external source of nitrogen. The cells were aerated in tubes for from 24 to 30 hr. on a reciprocal shaker at 30°. Ten different tubes of galactose-treated and of untreated cells were plated on galactose agar plates. The large (fermenter) colonies appearing on the plates were counted on the fifth, sixth and seventh days; the results are presented in Fig. 3. After 5 days the ten plates inoculated with cells pretreated with galactose buffer contained a total of ninety-eight colonies, while the ten plates inoculated with cells pretreated with buffer alone contained a total of thirty-six colonies. The fact that the pretreatment was carried on in the absence of an external source of nitrogen excluded selection and growth as complicating factors. The data show the characteristic low variance. The difference in the number of mutants obtained on the diagnostic plates following the different pretreatments cannot be ascribed to the difference in the number of cells plated. Plate counts of the viable cells, made on glucose medium as each sample was taken, revealed that both the plain buffer and the galactose buffer tubes contained 2×10^6 cells/ml. Remarkably few cells died during the treatments. Cells from both test and control were examined with a modified methylene blue dead cell stain (Townsend & Lindegren, 1953) with the following results: the percentages of dead cells in six glucose tubes were respectively, 2·9, 7·4, 8·1, 10·3, 1·9, 5·7; in seven galactose tubes, 10·7, 14·1, 11·8, 7·2, 11·5, 7·5, 8·9. Nine other experiments were run at different times, each involving approximately the same number of plates but using various concentrations of cells. Each showed a low variance and confirmed the findings described above.

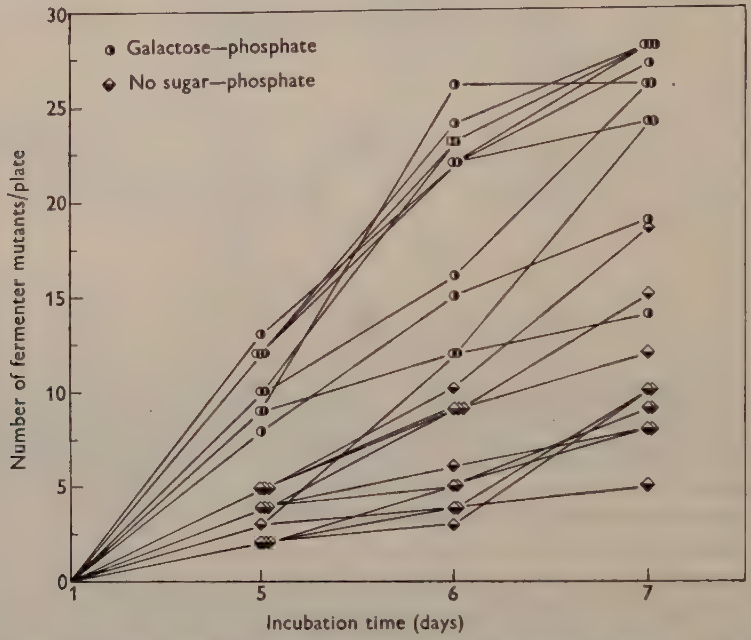


Fig. 3. The total number of large (galactose-fermenter) colonies appearing on separate galactose test plates inoculated from two series of ten flasks in which non-fermenter cells were pretreated (*a*) with galactose in buffer, and (*b*) with buffer without sugar. The plates were incubated at 30° and counted after the first, fifth, sixth and seventh days.

Table 5 describes another experiment in which glucose- and galactose-treated cells were planted on galactose agar and the number of mutant colonies counted after 6, 8 and 10 days. Instead of using complete medium, the treated cells were mixed with molten galactose-defined medium agar. In this experiment the numbers of dead cells in both series approximated 10%. In spite of the fact that the defined medium, which tends to diminish the number of divisions occurring on the plate, was used, the same difference was found between plates seeded with glucose- and galactose-treated cells.

Table 5. *The total number of large (galactose-fermenter) colonies appearing on separate galactose test plates inoculated from twenty different tubes in which non-fermenter cells were kept for 48 hr. in (a) phosphate buffer with 2% galactose and (b) phosphate buffer with 2% glucose*

Solutions contained 0·05% $MgSO_4.7H_2O$. The plates were incubated at 30° and counted after the 1st, 6th, 8th and 10th days.

Pretreatment		Days				Pretreatment		Days			
(48 hr.)	Sample	1	6	8	10	(48 hr.)	Sample	1	6	8	10
Galactose in phosphate buffer	1	0	5	8	11	Glucose in phosphate buffer	1	0	10	16	16
	2	0	4	6	6		2	0	4	8	9
	3	0	6	9	9		3	0	7	9	9
	4	0	3	5	7		4	0	9	9	10
	5	0	5	12	14		5	0	3	11	11
	6	0	5	20	21		6	0	8	11	11
	7	0	12	13	15		7	0	8	12	14
	8	0	3	4	14		8	0	12	13	13
	9	0	9	12	12		9	0	7	12	13
	10	0	11	13	13		10	0	11	20	21
Total		0	63	102	112			0	79	121	127

Mutability of the non-fermenter allele in dividing and non-dividing cells

Table 6 records the frequency distributions of galactose fermenter colonies on galactose plates seeded with similar cultures grown as follows: (*a*) full nutrient medium containing 2% glucose (Exp. 12*a*); (*b*) full nutrient medium containing 2% reagent galactose which has as impurities *c.* 0·3% glucose and 0·3% ethanol (Exp. 14*b*); and (*c*) Burkholder's (1943) medium containing 1% glucose + 1% galactose (Exp. 16*a*). In Exps. 14*a* and *b* the growth tubes were incubated for 5 days, while in Exp. 16*a*, the growth tubes were incubated for only 48 hr. The contents of each tube were mixed in a Petri dish with 15 ml. molten 2% galactose nutrient agar. The plates were counted after 5 days.

The number of large (galactose fermenter) colonies appearing on the galactose plates which had been seeded with the cells grown in the absence of galactose varied from 5 to 30, showing the characteristic low variance (Exp. 14*a*).

In Exp. 14*b* the glucose was all consumed long before the end of the incubation period, thus allowing several days' exposure to galactose in the absence of growth. The number of large colonies appearing on the galactose plates which were seeded with cultures that had been grown in the impure galactose showed an exceedingly high variance. Two-thirds of the cultures produced plates containing more than 30 colonies/plate, showing that mutation had occurred in the growth tube before plating. The fact that it is possible to set up conditions under which a high variance is observed proves that the characteristic low variance is not due to the suppression or crowding of the mutant cells on the galactose plates.

The plates seeded with the cells grown in the tubes containing equal amounts of glucose and galactose showed a low variance. In this experiment the growth tubes were incubated for only 48 hr. before seeding the galactose agar plate, and the cells had just completed the logarithmic phase. The presence of galactose produced no effect because the cells were still dividing at the time of transfer to the galactose agar. Galactose apparently cannot exert its mutagenic action on actively growing cells.

Table 6. *The effect of the presence of galactose in the growth tube on frequency distributions of galactose fermenter colonies appearing on galactose agar plates*

Exp. ...	14*a*	16*a*	14*b*
No. of cultures ...	56	50	53
Vol. of cultures (ml.)...	5·5	2·1	5·5
Vol. of samples (ml.)...	5·0	2·0	5·0
		No. of cultures	
No. of GA colonies			
0	0	0	0
1	0	0	0
2	0	0	0
3	0	1	0
4	0	0	0
5	1	1	0
6–10	18	26	0
11–15	28	18	1
16–20	6	4	3
21–25	0	0	5
26–30	3	0	6
31–500	0	0	18
500	0	0	20
Mean	12·82	10·52	—
Variance	20·51	18·59	—

Appearance of fermenter on galactose agar

When a rapid fermenter mutant is planted on galactose agar, it forms a large colony in less than 3 days, whether it is planted together with large or small numbers of non-fermenter cells. When non-fermenter cells grown in glucose are planted on galactose agar, the first count of the fermenter colonies is made on the fifth day. On the third day distinct tiny fermenter colonies make their first appearance and become large and easily counted on the fifth day. The fact that fermenter cells form large colonies before the third day is another piece of evidence supporting the view that they are absent from glucose grown cultures of the non-fermenter.

We are indebted to the following colleagues for the opportunity to discuss various phases of this problem: Mr E. Shult, Dr A. M. Mark, Mr A. Sarachek, Dr B. D. Mundkur and Dr A. L. Sheffner. This work was supported by research grants from the Southern Illinois University, The National Cancer Institute of the National Institutes of Health, Public Health Service, and Anheuser-Busch, Inc.

REFERENCES

Burkholder, P. R. (1943). Vitamin deficiencies in yeast. *Amer. J. Bot.* **30**, 206.

James, A. P. (1952). An exceptionally frequent induced genetic change in yeast. (Abstracts) Papers presented at the 1952 meetings of the Genetics Society of America. *Genetics*, **37**, 592.

Lea, D. E. & Coulson, C. A. (1949). The distribution of the numbers of mutants in bacterial populations. *J. Genet.* **49**, 264.

Lindegren, C. C. (1949). *The Yeast Cell, Its Genetics and Cytology.* St Louis, Mo. Educational Publishers, Inc.

Lindegren, C. C. (1953). Concepts of gene-structure and gene-action derived from tetrad analysis of *Saccharomyces. Experientia*, **9**, 75.

Lindegren, C. C. & Palleroni, N. J. (1952). Absence of the preadaptive utilization of galactose by yeasts. *Nature, Lond.* **169**, 879.

Luria, S. E. & Delbrück, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics*, **28**, 491.

Mundkur, B. D. (1952). Long-term adaptation to galactose by yeast. *Genetics*, **37**, 484.

Mundkur, B. D. & Lindegren, C. C. (1949). An analysis of the phenomenon of long-term adaptation to galactose by *Saccharomyces. Amer. J. Bot.* **36**, 722.

Newcombe, H. B. (1949). Origin of bacterial variants. *Nature, Lond.* **164**, 150.

Novick, A. & Szilard, L. (1950). Experiments with the chemostat on spontaneous mutations of bacteria. *Proc. nat. Acad. Sci., Wash.* **36**, 708.

Sheffner, A. L. & Lindegren, C. C. (1952). Adaptation to the prefermentative oxidation of galactose. *J. Bact.* **64**, 423.

Spiegelman, S., De Lorenzo, W. F. & Campbell, A. M. (1951). A single cell analysis of the transmission of enzyme forming capacity in yeast. *Proc. nat. Acad. Sci., Wash.* **37**, 513.

Spiegelman, S., Sussman, R. R. & Pinska, E. (1950). On the cytoplasmic nature of 'long-term' adaptation in yeast. *Proc. Nat. Acad. Sci., Wash.* **36**, 591.

Townsend, G. F. & Lindegren, C. C. (1953). Structures in the yeast cell revealed in wet mounts. *Cytologia* (in the Press).

Winge, O. & Roberts, C. (1948). Inheritance of enzymatic characters in yeasts and the phenomenon of long-term adaptation. *C.R. Lab. Carlsberg*, **24**, 263.

(*Received* 18 *June* 1953)

Salton, M. R. J. (1953). *J. gen. Microbiol.* **9**, 512–523.

Cell Structure and the Enzymic Lysis of Bacteria

By M. R. J. SALTON*

Department of Bacteriology, University of California, Berkeley, California

SUMMARY: Untreated cells of three Gram-negative and four Gram-positive bacteria were resistant to lysis by crude and crystalline trypsin. The resistance of the Gram-negative organisms to lysis by trypsin was abolished by heating suspensions for 5 min. at 100°; the rates and extent of lysis of the three organisms by crude trypsin were comparable. After maximal lysis of heated suspensions of *Bacterium coli* and *Pseudomonas fluorescens* by crude trypsin, examination of the residual material in the electron microscope showed that cell walls were the principal components. When heated suspensions of *Micrococcus lysodeikticus*, *Bacillus megaterium* and *Staphylococcus aureus* were incubated with crude or crystalline trypsin, slow lysis occurred and the extent of lysis was less than that observed for the Gram-negative bacteria. Electron microscopic examination of trypsin-lysed cells of *B. megaterium* showed incomplete digestion of cytoplasmic contents. Heated and autoclaved cells of *Streptococcus faecalis* were resistant to digestion with trypsin.

Cell-wall suspensions of *B. megaterium* were rapidly lysed on incubation with crystalline lysozyme. Heated cells of *M. lysodeikticus* were only partially lysed by lysozyme, but the subsequent addition of trypsin resulted in a rapid dissolution of the cell bodies. Although treatment of heated *B. megaterium* cells with lysozyme resulted in turbidity increases, the enzyme completely digested the cell wall leaving coagulated protoplasmic bodies. The protoplasmic bodies were rapidly lysed by crude trypsin.

One of the most elegant methods of studying the nature and location of surface components of the bacterial cell has been that afforded by the action of certain enzymes. Capsules and other superficial layers of living bacterial cells have been removed enzymically without impairing cell viability. Enzymic degradation of type III pneumococcus polysaccharide capsule (Dubos & Avery, 1931) and removal of the M-protein antigen from haemolytic streptococci (Lancefield, 1943) were achieved without death of the organisms. However, certain enzymes such as lysozyme (Fleming, 1922) bring about the death and rapid dissolution of susceptible bacteria. It has been known for some time that lysozyme digests a cellular component essential for the maintenance of the cell's morphological structure (Epstein & Chain, 1940). That lysozyme effects a dissolution of the rigid cell-wall structure of *Micrococcus lysodeikticus* has now been established directly by studying the action of crystalline lysozyme from egg-white on the separated walls of this organism (Salton, 1952*a*). At least one other enzyme system is capable of degrading cell walls of bacteria other than those attacked by lysozyme. Maxted (1948) showed that the enzymes produced by a *Streptomyces* sp. lysed suspensions of living β-haemolytic streptococci. Rapid lysis of turbid suspensions of separated cell walls of *Strep. pyogenes* by an enzyme preparation from *Streptomyces* sp. (obtained by Maxted's procedure) was reported by Salton (1952*b*). McCarty (1952*a*, *b*) investigated the lysis of group A haemolytic streptococci by the extracellular enzymes of *Streptomyces albus* and concluded that the same enzyme fractions which were effective in lysing intact cells of group A streptococci also dissolved the isolated cell walls. Thus enzymes such as lysozyme and those of *S. albus* which are capable of lysing intact organisms, appear to have a primary lytic action upon the cell walls.

---

* Present address: Department of Biochemistry, Cambridge, England.

The nature and properties of the bacteriolytic substances produced by various micro-organisms have been the subject of many investigations (Waksman, 1947; Welsch, 1947; Jones, Swallow & Webb, 1948; Muggleton & Webb, 1952; Tai & van Heyningen, 1951; Born, 1952). Both heat-killed bacteria and living organisms dispersed in agar have been used extensively for the detection and isolation of soil micro-organisms which produce bacteriolytic enzymes. However, it has been difficult to judge whether lytic activities were due to a primary attack on the bacterial cell walls or to other processes involving secondary lysis by proteolytic enzymes. There can be little doubt as to the importance of proteolytic enzymes in the lysis of heated bacteria. Indeed, as early as 1909, Kantorowicz reported that Gram-negative bacteria became susceptible to lysis by trypsin after they had been heated at 80°, whereas Gram-positive organisms were resistant to trypsin lysis even after boiling. The loss of resistance to digestion by trypsin which bacterial cells undergo on heating and the greater susceptibility of heat-killed, Gram-negative bacteria to lysis by proteolytic enzymes have never been adequately explained. Observations showing that heat-treatment resulted in rupture of the cell walls of certain bacteria (Salton & Horne, 1951*a*) suggested that susceptibility of heated bacteria to lysis by proteolytic enzymes may be governed by the nature of the cell wall and the effects of heat thereon. Indeed, the cell walls of Gram-negative bacteria generally showed extensive rupture on heating at temperatures above 75°, whereas the walls of Gram-positive bacteria showed little or no rupture even on heating at 100° (Salton, 1951). The present investigations were undertaken to determine the fate of the cell walls during enzymic lysis of heated bacteria, thereby to obtain some indication of the suitability of using heat-killed bacteria for the isolation of cell-wall decomposing micro-organisms.

METHODS

Organisms. The following organisms were used: *Bacterium coli* strain B; *Pseudomonas fluorescens* strain A 3.12; *Spirillum serpens* (ATCC 11330); *Bacillus megaterium* strain KM; *Micrococcus lysodeikticus* (NCTC 2665); *Streptococcus faecalis* (NCTC 6782) and *Staphylococcus aureus* (F.D.A. strain of the U.S. Department of Agriculture).

Media, growth conditions and harvesting. The three Gram-negative organisms were grown in liquid medium consisting of (%, w/v): 0·5 Bacto yeast extract; 0·3 Bacto peptone; 0·1 % K_2HPO_4. *B. megaterium* was grown in 5 % Bacto peptone broth, *Staph. aureus* in nutrient broth and the two other Gram-positive bacteria in a liquid medium containing 2 % Bacto-tryptone, 0·1 % yeast extract and 1 % glucose. Flasks of medium were inoculated with 24 hr. cultures and incubated at 30° with mechanical shaking for 16 hr. Cells were harvested from the medium by centrifugation and washed twice with distilled water.

Enzyme preparations. Crystalline egg-white lysozyme (Armour), crude trypsin, crystalline trypsin (containing *c.* 50% $MgSO_4$) and crystalline salt-free pancreatic ribonuclease were used. They were dissolved in 0·1 M-phosphate buffers. Suspended material in the crude trypsin solutions was removed by centrifugation. All enzyme concentrations quoted in the results refer to final concentrations (w/v basis).

Turbidimetric study of lysis. The lytic action of the enzymes was studied with the following types of preparations: (1) untreated bacteria suspended in 0·1 M-phosphate buffers; (2) cell suspensions which had been subjected to heating at 100°; thick suspensions of bacteria were pipetted into large volumes of distilled water held in a water-bath at 100°, and the suspensions were heated at 100° for 5 min. Heated suspensions were cooled, centrifuged, and the deposits washed once with distilled water and finally suspended in 0·1 M-phosphate buffers; (3) cell walls of *B. megaterium* prepared by disintegration of the cells (10% wet-weight suspension of bacteria) in the Raytheon 9 Kc. Magnetostriction Oscillator for 10 min. The subsequent steps in the preparation of the cell walls were those outlined by Salton & Horne (1951*b*); cell walls were finally suspended in 0·1 M-phosphate buffer.

The Klett-Summerson photoelectric colorimeter with a green filter (no. 54, spectral range 500–570 mμ.) was used throughout the investigation for measuring turbidities. Suspension densities of untreated bacteria, heated cells and cell walls in 0·1 M-phosphate buffers were adjusted to give final Klett readings of *c.* 400, corresponding to dry weights of *c* 1·5 mg. cells or cell walls/ml.; 9·9 ml. of each suspension were pipetted into Klett tubes which were then placed in a constant-temperature water bath at 37°. To these tubes 0·1 ml. of enzyme preparation in buffer or 0·1 ml. of buffer (control tubes) was added and the contents thoroughly mixed. Tubes were shaken each time before reading at various intervals in the photoelectric colorimeter.

Preparation of material for electron microscopy. Suspensions were centrifuged for 15 min. at 10,000 r.p.m. after which the deposits were washed once with phosphate buffer and three times with distilled water on the high-speed centrifuge. The material was finally suspended in distilled water and diluted suitably for placement on electron microscope grids.

RESULTS

Lysis experiments with Gram-negative bacteria

Untreated suspensions of *Bact. coli*, *Ps. fluorescens* and *Sp. serpens* in phosphate buffer were incubated for 2 hr. at 37° with: (1) crude trypsin, 1 mg./ml., pH 7·6; (2) crystalline trypsin, 100 μg./ml., pH 7·6; (3) crystalline ribonuclease, 50 μg./ml. pH 7·6; (4) crystalline lysozyme concentrations of 100 μg. and 1 mg./ml., pH 6·2. Turbidity readings of control and enzyme-treated suspensions taken at intervals over the incubation period showed no significant differences. Addition of lysozyme (1 mg./ml.) to bacterial suspensions brought about agglutination of the cells. The cells could be redispersed merely by shaking the tubes, and no change in the turbidities of suspensions was observed. Agglutination of bacterial cells by lysozyme has often been encountered in the past (see Thompson, 1940).

The addition of crude or crystalline trypsin to heat-killed suspensions of *Bact. coli* in phosphate buffer (pH 7·6) brought about a rapid decrease in turbidity; no such change occurred in control suspensions of heated cells. Incubation of suspensions of heated *Bact. coli* with crystalline ribonuclease resulted in a small (*c.* 5 %) but consistent decrease in turbidity within 15 min. at 37°, no further change being apparent on continued incubation. The course of lysis of suspensions of heated *Bact. coli* by crude trypsin (1 mg./ml., pH 7·6), crystalline trypsin (100 µg./ml., pH 7·6) and crystalline ribonuclease (50 µg./ml., pH 7·6) is illustrated in Fig. 1. Similar lysis curves were also obtained when crude trypsin was added to suspensions of heated *Ps. fluorescens* and *Sp. serpens*. Typical results of lysis experiments with *Ps. fluorescens* and *Sp. serpens* are compared with those for *Bact. coli* in Table 1, where the lytic activity of trypsin is expressed as percentage lysis, i.e. percentage decrease in turbidity.

Table 1. *Lysis of heat-killed Gram-negative bacteria by crude trypsin (1 mg./ml.,* 0·1 M-*phosphate buffer,* pH 7·6) *at* 37°

Time of incubation (min.)	Percentage lysis		
	Bact. coli	*Ps. fluorescens*	*Sp. serpens*
0	0	0	0
5	45·7	32·4	49·1
10	59·9	50·5	67·9
15	69·1	61·3	78·0
20	74·1	68·5	83·8
25	76·0	73·5	86·7
30	79·0	77·1	88·8
35	81·0	79·3	89·5
40	81·5	81·0	90·0
45	82·0	82·0	90·8
50	82·5	83·0	91·0
55	82·7	83·9	91·2
60	83·1	84·2	91·2

When lysozyme was added to suspensions of heated *Bact. coli* and *Ps. fluorescens* in phosphate buffer (pH 6·2) at 37° (final concentration of lysozyme, 1 mg./ml.) turbidity increases of 20 and 30 % respectively were observed. The addition of lysozyme resulted in an immediate increase in turbidity which remained unchanged for periods of up to 12 hr. It appeared likely that the basic properties of the lysozyme, rather than its enzymic capabilities, may have been responsible for the increase in turbidities. This possibility was strengthened by the observation of a similar phenomenon when the protamine, salmine, was added to suspensions of heated *Bact. coli* in phosphate buffer, pH 6·2. The addition of salmine (final concentration of 1 mg./ml.) to heated *Bact. coli* resulted in an immediate 13 % increase in turbidity, and no further change occurred on incubation for 12 hr. at 37°.

The results presented in Table 1 have shown that lysis of heated suspensions of *Bact. coli* and *Ps. fluorescens* by crude trypsin was virtually completed in 1 hr., and that the residual material accounted for 17 and 16 % of the original turbidities of the suspensions. An examination of the material left after maximal clearing (2 hr. at 37°) of the suspensions by crude trypsin was carried out with the aid of the electron microscope. The residues from trypsin-lysed *Bact. coli* and *Ps. fluorescens* are shown in Pl. 1, figs. 1 and 2, respectively. It is evident that the cell walls constitute the principal component of the residues from these organisms. In addition to the cell walls of *Ps. fluorescens* (Pl. 1, fig. 2) there would appear to be another layer of material which has also resisted digestion with trypsin.

Lysis experiments with Gram-positive bacteria

Micrococcus lysodeikticus. Turbidity measurements performed on suspensions of unheated cells of *M. lysodeikticus* in phosphate buffer (pH 7·6) containing either crude trypsin (1 mg./ml.), crystalline trypsin (100 μg./ml.) or crystalline ribonuclease (50 μg./ml.) showed no differences from control suspensions in buffer alone, when incubated for 2 hr. at 37°. Unheated cells of this organism in phosphate buffer (pH 6·2) were rapidly lysed on incubation with lysozyme at 37°. The lysis curve obtained with 5 μg. lysozyme/ml. is shown in Fig. 2 (curve 2). Lysis of *M. lysodeikticus* with a high concentration of lysozyme (1 mg./ml.) resulted in only a 30 % decrease in turbidity in 15 min. (compared to 94 % with 5 μg. lysozyme/ml.) and no further lysis occurred on incubation for 1 hr. at 37°.

Lysis of heated cells of *M. lysodeikticus* was investigated under the following conditions: (1) incubation with lysozyme, 5 μg./ml., pH 6·2, 60 min.; (2) treatment with lysozyme as in (1) for 30 min., followed by sedimentation of the cells, resuspension in phosphate buffer at pH 7·6 and incubation with crude trypsin (1 mg./ml.); (3) incubation with crude trypsin, 1 mg./ml., pH 7·6, 60 min.; (4) control suspensions in phosphate buffers, pH 6·2 and 7·6. Experiments were performed at 37°, and the course of lysis followed turbidimetrically. The results are presented in Fig. 2 which shows for comparison the lysis of unheated *M. lysodeikticus* by lysozyme. A lysis curve similar to that shown for crude trypsin (Fig. 2, curve 5) was obtained when heated cells were incubated with crystalline trypsin (100 μg./ml.) and the percentage lysis in 1 hr. (37°) was 26 % compared to 28 % with crude trypsin (1 mg./ml.). Incubation of heated cells with crystalline ribonuclease (50 μg./ml., pH 7·6) resulted in a 4·5 % decrease in turbidity within 15 min. at 37°; no further change on incubation for 1 hr. could be detected.

Suspensions of heated *M. lysodeikticus* examined in the electron microscope before and after treatment with 5 μg. lysozyme/ml. (pH 6·2, 37°) for 60 min. showed that the turbidity decrease (Fig. 2, curve 3) occurred concomitantly with digestion of the cell walls.

Bacillus megaterium. Rapid dissolution of the separated cell walls of *M. lysodeikticus* by lysozyme, was demonstrated in an earlier communication (Salton, 1952*a*). Cell walls of *B. megaterium* were prepared (Pl. 1, fig. 3, shows a typical field of the preparation) in order to determine whether lysozyme also effected lysis of the walls of this organism. Suspensions of cell walls in phosphate buffer (pH 6·2) were incubated with lysozyme concentrations of 50 μg. and 100 μg./ml. at 37°, and turbidity measurements were made on control and lysozyme-treated suspensions. The progress of lysis of the walls by lysozyme is shown in Fig. 3. No change in turbidity resulted from incubation of cell walls in phosphate buffer (pH 7·6) with crude trypsin (1 mg./ml.) or crystalline trypsin (100 μg./ml.) for 1 hr. at 37° (Fig. 3).

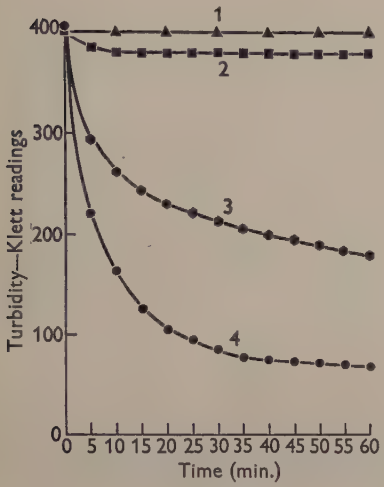


Fig. 1

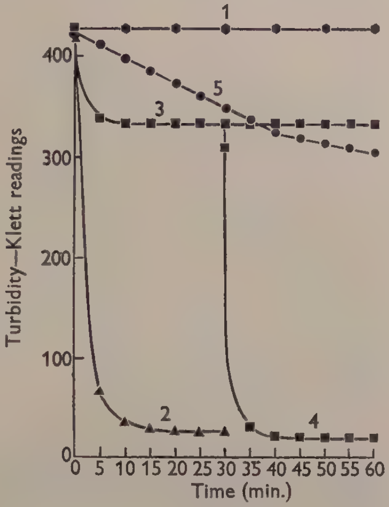


Fig. 2

Fig. 1. Lysis of heat-killed *Bact. coli* in 0·1 M-phosphate buffer at 37°. Curve 1, heated cells in buffer (pH 7·6) alone; curve 2, suspensions incubated with 50 μg. crystalline ribonuclease/ml., pH 7·6; curve 3, treatment with crystalline trypsin—100 μg./ml., pH 7·6; curve 4, treatment with crude trypsin—1 mg./ml., pH 7·6.

Fig. 2. Enzymic lysis of *M. lysodeikticus* in 0·1 M-phosphate buffer at 37°. Curve 1, untreated and heated cells suspended in buffer (pH's 6·2 and 7·6) alone; curve 2, lysis of unheated cells with crystalline lysozyme—5 μg./ml., pH 6·2; curve 3, heated cells incubated with crystalline lysozyme—5 μg./ml., pH 6·2; curve 4, heated cells incubated with lysozyme as in curve 3 for 30 min., followed by sedimentation of the cells, resuspension in buffer at pH 7·6 and incubation with crude trypsin—1 mg./ml.; curve 5, lysis of heated cells with crude trypsin—1 mg./ml., pH 7·6.

No lysis of unheated *B. megaterium* cells was detected on incubation with crude trypsin, crystalline trypsin or crystalline ribonuclease under similar conditions to those used with other organisms. Lysozyme (100 μg./ml.) rapidly lysed untreated cells as shown in Fig. 4 (curve 1) but the extent of lysis was less than that observed for *M. lysodeikticus*. The addition of crude trypsin (1 mg./ml., final concentration) to suspensions of lysozyme-lysed *B. megaterium* resulted in a rapid fall in turbidity (Fig. 4, curve 2) even at pH 6·2, a lower pH than that normally employed for tryptic digestion of proteins.

Heated suspensions of *B. megaterium* showed no lysis when treated with lysozyme (100 μg./ml., pH 6·2). Indeed, an immediate increase in turbidity was observed and the phenomenon is illustrated in Fig. 4, curve 4. Suspensions of heated *B. megaterium* which had been incubated with 100μg. lysozyme/ml. (pH 6·2) for 60 min. at 37° were examined in the electron microscope. The appearance of heated cells is shown in Pl. 1, fig. 4, Pl. 2, fig. 5, and the heated cells treated with lysozyme are illustrated in Pl. 2, fig. 6. Although the turbidity of heated suspensions of *B. megaterium* was increased on incubation with lysozyme, examination in the electron microscope clearly showed the complete removal of the cell walls. Many short rods with square ends were observed in the heated *B. megaterium* preparation which had been treated with lysozyme. These 'short rods' are undoubtedly similar to the 'small bacilli' observed by Welshimer & Robinow (1949) in both formaldehyde-treated and heated *B. megaterium* preparations, treated with lysozyme. There can be little doubt that the dissolution of the cross-walls of multicellular units of *B. megaterium* by lysozyme is responsible for (*a*) an increase in turbidity due to the breakdown of large multicellular rods into a greater number of 'small bacilli' and (*b*) the appearance of many short rods with characteristic square ends. Heated suspensions of *B. megaterium* were treated with 100μg. lysozyme/ml. for 30 min. at 37° and the coagulated protoplasmic bodies corresponding to those illustrated in Pl. 2, fig. 6, were sedimented and the deposits resuspended in phosphate buffer, pH 7·6 and incubated with crude trypsin (1 mg./ml.) at 37°. Under these conditions rapid lysis occurred and the course of lysis is shown in Fig. 4, curve 5.

Incubation of heated cells with crude trypsin resulted in a low, steady rate of lysis as shown in Fig. 4, curve 6. The extent and rate of lysis of heated *B. megaterium* with crystalline trypsin (100μg./ml.) were comparable to those shown for crude trypsin (1 mg./ml.) in Fig. 4 (curve 6). The average percentage lysis in 1 hr. at 37° was 10·5 % for crystalline trypsin (100μg./ml.) compared to 12·1 % for crude trypsin (1 mg./ml.) and on further incubation for a total of 3 hr. at 37° the percentage lysis had increased to 30 and 36 % respectively. Heated cells of *B. megaterium* were examined in the electron microscope after incubation with 1 mg. crude trypsin/ml. in phosphate buffer (pH 7·6) for 2 hr. at 37° (lysis, *c.* 24 %). Pl. 2, figs. 7, 8, show the typical appearance of the trypsin-lysed cells. Despite the intact appearance of the cell walls of heated cells and of unheated trypsin-lysed cells, a comparison of the heated cells (Pl. 1, fig. 4, Pl. 2, fig. 5) with those incubated with crude trypsin demonstrates the loss of electron-dense material due to digestion of the protoplasmic contents.

Heat-killed *B. megaterium* incubated with crystalline ribonuclease (50μg./ml.) in phosphate buffer, pH 7·6, at 37°, consistently showed a reduction in turbidity of *c.* 4 % within 15 min., after which no further change in turbidity occurred on incubation for periods of up to 3 hr.

Staphylococcus aureus. Unheated cells of *Staph. aureus* suspended in phosphate buffer were incubated with several concentrations of lysozyme (50μg., 100μg., and 1 mg./ml., pH 6·2) and crude trypsin (1 mg./ml., pH 7·6). No differences between turbidities of control, lysozyme-treated and crude trypsin-treated suspensions could be detected on incubation for 2 hr. at 37°.

Lysis of heated cells was investigated by incubating suspensions with crude trypsin (1 mg./ml.), lysozyme (100 μg./ml.) and lysozyme (100 μg./ml., pH 6·2) followed by treatment with crude trypsin (1 mg./ml., pH 7·6). The results are summarized in Table 2. The enhanced trypsin lysis of heated cells of *Staph. aureus* that have been previously exposed to lysozyme is in agreement with the results reported by Webb (1948).

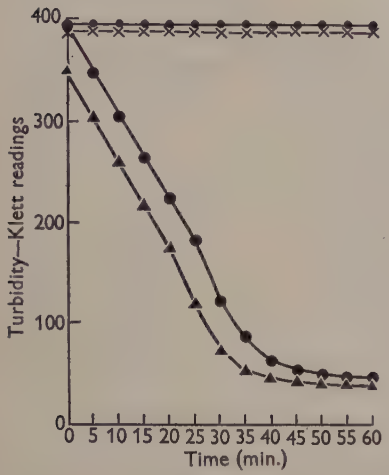


Fig. 3

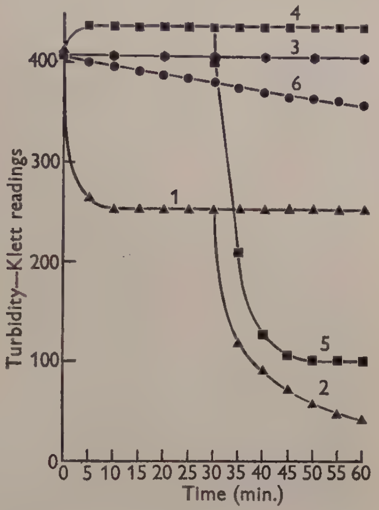


Fig. 4

Fig. 3. Lysis of *B. megaterium* cell walls in 0·1 M-phosphate buffer at 37°. ●—●, cell walls suspended in buffer (pH's 6·2 and 7·6) alone; ×—×, cell walls incubated with crystalline trypsin—100 μg./ml., pH 7·6; ●—●, cell walls incubated with 100 μg. crystalline lysozyme/ml., pH 6·2; ▲—▲, cell walls incubated with 50 μg. crystalline lysozyme/ml., pH 6·2.

Fig. 4. Enzymic lysis of *B. megaterium* in 0·1 M-phosphate buffer at 37°. Curve 1, lysis of unheated cells by crystalline lysozyme—100 μg./ml., pH 6·2; curve 2, unheated cells lysed with lysozyme as in curve 1 for 30 min. followed by the addition of crude trypsin (1 mg./ml. final concentration), pH 6·2; curve 3, heated cells suspended in buffer (pH's 6·2 and 7·6) alone; curve 4, heated cells incubated with 100 μg. crystalline lysozyme/ml., pH 6·2; curve 5, heated cells treated with 100 μg. crystalline lysozyme/ml. for 30 min. at pH 6·2 (as in curve 1) followed by sedimentation of the cells, resuspension in buffer at pH 7·6 and incubation with crude trypsin (1 mg./ml.); curve 6, heated cells incubated with crude trypsin—1 mg./ml., pH 7·6.

Streptococcus faecalis. There was no evidence of lysis of unheated suspensions of *Strep. faecalis* on incubation with crude trypsin (1 mg./ml., pH 7·6) or lysozyme (100 μg./ml., pH 6·2) for 2 hr. at 37°. Heated cells of this organism showed no lysis on incubation with crude trypsin (1 mg./ml.), nor was their turbidity affected by the presence of lysozyme (100 μg./ml.). Repeated attempts to detect lysis of heated cells of *Strep. faecalis* during extended periods of incubation at 37° with crude trypsin (1 mg./ml.) were without success. Indeed, cells autoclaved for 20 min. at 120°, centrifuged and resuspended in phosphate buffer (pH 7·6) were not lysed by crude trypsin (1 mg./ml.) or crystalline trypsin (100 μg./ml.) on incubation at 37° for up to 12 hr. The resistance of heated *Strep. faecalis* to digestion with trypsin is similar to that recently reported for group A streptococci by McCarty (1952*a*, *b*).

Table 2. *Enzymic lysis of heated* (5 *min. at* 100°) Staph. aureus *suspensions*

(0·1 M-phosphate buffer, temperature of incubation 37°)

Time of incubation (min.)	Percentage lysis		
	Crude trypsin (1 mg./ml.; pH 7·6)	Crystalline lysozyme (100 μg./ml.; pH 6·2)	Pre-treated with Crystalline lysozyme (100 μg./ml., pH 6·2 for 1 hr.); incubated with crude trypsin (1 mg./ml., w/v; pH 7·6)
5	2·5	+10·7*	11·4
10	4·9	+10·7*	18·2
15	7·4	+10·7*	22·7
20	9·9	+10·7*	26·1
25	12·4	+10·7*	28·6
30	13·6	+10·7*	30·7
35	14·8	+10·7*	31·5
40	16·0	+10·7*	32·3
45	17·3	+10·7*	33·2
50	17·3	+10·7*	34·1
55	18·5	+10·7*	34·8
60	18·5	+10·7*	35·5

* Increased turbidity in the presence of lysozyme.

DISCUSSION

There can be little doubt that the resistance of living bacteria to digestion by proteolytic enzymes, such as trypsin, is largely due to the nature of their cell walls. The resistance of bacterial cell walls to dissolution by trypsin has been adequately demonstrated by the use of this enzyme in certain stages of the preparation of cell walls (Weidel, 1951; McCarty, 1952*b*). The M-protein antigen retained by the cell walls of mechanically disintegrated *Strep. pyogenes* has been removed with trypsin without affecting the rigidity or appearance of the cell walls (Salton, 1952*b*, 1953). Furthermore, the turbidity of separated cell walls of *B. megaterium* was unaffected by incubation with crude or crystalline trypsin (see Fig. 3).

The complete loss of resistance to lysis with trypsin that Gram-negative bacteria undergo on heating can now be explained in terms of damage to the bacterial cell wall. The walls of Gram-negative bacteria are ruptured on heating (Salton & Horne, 1951*a*) thereby exposing the coagulated protoplasmic contents of the cell to the direct action of proteolytic enzymes. Earlier work (Kantorowicz, 1909; Tai & van Heyningen, 1951) showed that Gram-negative bacteria become highly susceptible to tryptic digestion on heating at temperatures of *c*. 70–80°, and this coincides remarkably well with the temperature range (70–75°) within which cell wall rupture or damage first becomes apparent (Salton & Horne, 1951*a*). In the absence of direct microscopical evidence it might have been argued that heating renders both the cell walls and protoplasmic contents of Gram-negative bacteria susceptible to digestion with trypsin. However, cell walls are the principal trypsin-resistant structures found in the residues from heated suspensions of certain

Gram-negative bacteria that have been lysed with trypsin (see Pl. 1, figs. 1, 2). Salton (1952*b*, 1953) found that the cell walls of several Gram-negative bacteria contained considerably more lipid than do any of the walls of Gram-positive organisms, and that the cell walls of Gram-negative and Gram-positive bacteria differ in their amino-acid constitution. Whether the susceptibility of the cell walls of Gram-negative bacteria to rupture or damage on heating is due to the high lipid content or the nature of the protein component cannot be decided at the moment. A detailed comparison of the constitution of cell walls prepared by heat-treatment rupture or by mechanical disintegration (Salton & Horne, 1951*b*) may provide evidence on this point. It is also conceivable that a melting of the lipid components, instead of actual rupture of the cell walls on heating Gram-negative bacteria, would equally render the coagulated protoplasm vulnerable to digestion with proteolytic enzymes.

There is general agreement that heat-killed Gram-positive organisms are more resistant to lysis with proteolytic enzymes than are heated Gram-negative bacteria. Not all heated Gram-positive bacteria are completely resistant to lysis with crystalline trypsin, e.g. pneumococci studied by Dubos (1937) and certain organisms studied in the present investigation. Although very little or no cell-wall rupture can be detected on heating Gram-positive organisms (Salton & Horne, 1951*a*; Salton, 1951; and Pl. 1, fig. 4, Pl. 2, fig. 5, of this paper) the cells have been modified sufficiently to allow the penetration of trypsin molecules with subsequent digestion of the coagulated protoplasmic contents (see Pl. 2, figs. 7, 8). The greater resistance of heat-killed Gram-positive bacteria to trypsin lysis does not appear to be a property of the coagulated protoplasm of these organisms, for it has been adequately demonstrated that enzymic degradation of the cell walls of heated bacteria greatly facilitates tryptic digestion. It is clear, therefore, that the resistance of heated Gram-positive bacteria to digestion by proteolytic enzymes is largely governed by the nature of the cell walls.

The evidence presented in this paper illustrates the unsuitability of heated bacterial cells as a substrate for the detection of enzymes which decompose cell walls. Extensive lysis of heated Gram-negative bacteria can be effected with the proteolytic enzyme, trypsin, without dissolution of the cell walls. The unsuitability of using heat-killed bacteria for the detection of enzymes which degrade cell walls is further shown by the action of lysozyme on heated *B. megaterium*. There is abundant evidence that enzymic lysis of suspensions of living organisms is correlated with the dissolution of the separated cell walls by the lytic enzyme. However, the production of lytic zones by the growth of soil micro-organisms on nutrient agar inoculated with living bacteria may well be the result of the combined action of bactericidal substances and of proteolytic enzymes. The isolation of micro-organisms which produce lytic zones on agar containing sufficient quantities of separated cell walls to give an opaque medium, would appear to offer favourable conditions for the selection of organisms capable of producing cell-wall decomposing enzymes.

I should like to express my thanks to Merck & Co. Inc., for the award of the Merck International Fellowship, and to Prof. R. Y. Stanier for his generous hospitality and interest in the investigations. I am most grateful to Dr Robley C. Williams for the preparation of the electron micrographs.

REFERENCES

Born, G. V. R. (1952). The extracellular bacteriolytic enzymes of a species of Streptomyces. *J. gen. Microbiol.* **6**, 344.

Dubos, R. J. (1937). The autolytic system of pneumococci. *J. exp. Med.* **65**, 873.

Dubos, R. J. & Avery, O. T. (1931). Decomposition of the capsular polysaccharide of pneumococcus type III by a bacterial enzyme. *J. exp. Med.* **54**, 51.

Epstein, L. A. & Chain, E. (1940). Some observations on the preparation and properties of the substrate of lysozyme. *Brit. J. exp. Path.* **21**, 339.

Fleming, A. (1922). On a remarkable bacteriolytic element found in tissues and secretions. *Proc. roy. Soc.* B, **93**, 306.

Jones, A. S., Swallow, A. J. & Webb, M. (1948). The exocellular bacteriolytic system of soil actinomyces. 1. The nature and properties of the lytic system. *Biochim. biophys. Acta*, **2**, 167.

Kantorowicz, A. (1909). Bakterien-Antifermente und Bakteriolyse. *Münch. med. Wschr.* **56**, 897.

Lancefield, R. C. (1943). Studies on the antigenic composition of group A hemolytic streptococci. *J. exp. Med.* **78**, 465.

Maxted, W. R. (1948). Preparation of streptococcal extracts for Lancefield grouping. *Lancet*, ii, 255.

McCarty, M. (1952*a*). The lysis of group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. I. Production and fractionation of the lytic enzymes. *J. exp. Med.* **96**, 555.

McCarty, M. (1952*b*). The lysis of group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. II. Nature of the cellular substrate attacked by the lytic enzymes. *J. exp. Med.* **96**, 569.

Muggleton, P. W. & Webb, M. (1952). The exocellular bacteriolytic system of soil actinomyces. II. A further investigation of the lytic system. *Biochim. biophys. Acta*, **8**, 431.

Salton, M. R. J. (1951). Some properties of the bacterial cell envelope. Thesis: University of Cambridge.

Salton, M. R. J. (1952*a*). Cell wall of *Micrococcus lysodeikticus* as the substrate of lysozyme. *Nature, Lond.* **170**, 746.

Salton, M. R. J. (1952*b*). The nature of the cell walls of some Gram-positive and Gram-negative bacteria. *Biochim. biophys. Acta*, **9**, 334.

Salton, M. R. J. (1953). Studies of the bacterial cell wall. IV. The composition of the cell walls of some Gram-positive and Gram-negative bacteria. *Biochim. biophys. Acta*, **10**, 512.

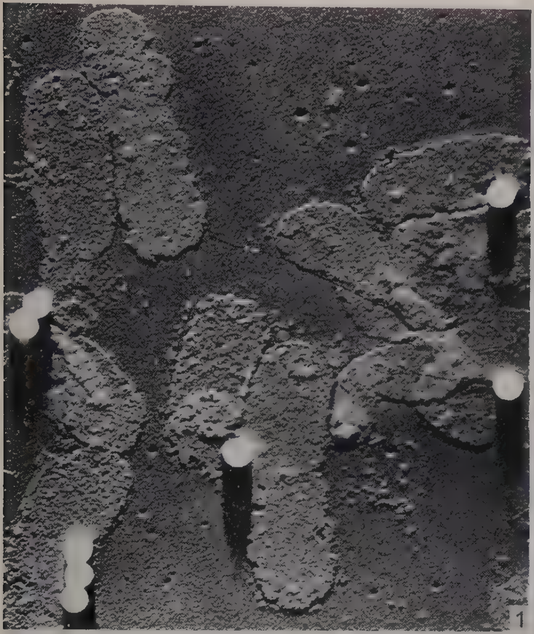
Salton, M. R. J. & Horne, R. W. (1951*a*). Studies of the bacterial cell wall. I. Electron microscopical observations on heated bacteria. *Biochim. biophys. Acta* **7**, 19.

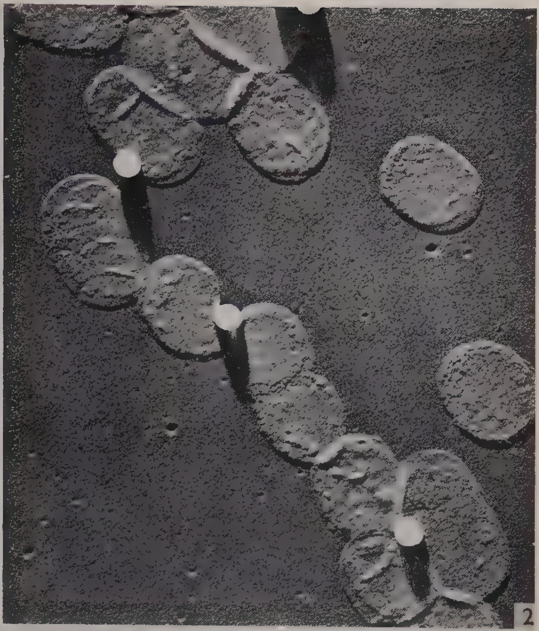
Salton, M. R. J. & Horne, R. W. (1951*b*). Studies of the bacterial cell wall. II. Methods of preparation and some properties of cell walls. *Biochim. biophys. Acta*, **7**, 177.

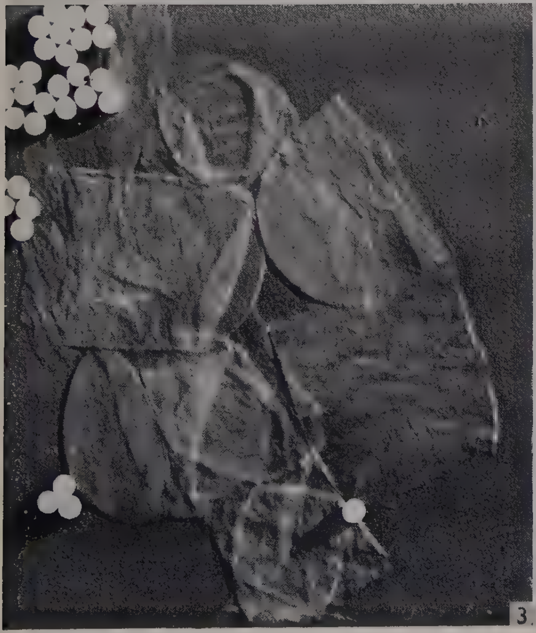
Tai, T. Y. & van Heyningen, W. E. (1951). Bacteriolysis by a species of Streptomyces. *J. gen. Microbiol.* **5**, 110.

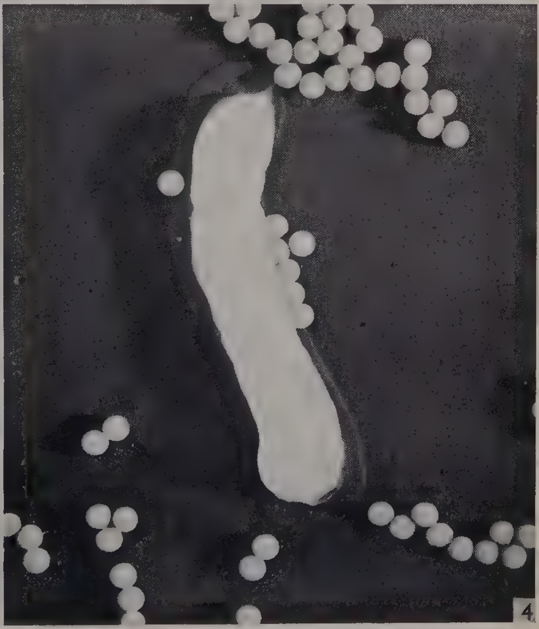
Thompson, R. (1940). Lysozyme and its relation to antibacterial properties of various tissues and secretions. *Arch. Path.* **30**, 1096.

Waksman, S. A. (1947). *Microbial Antagonism and Antibiotic Substances*, 2nd ed. New York: The Commonwealth Fund.

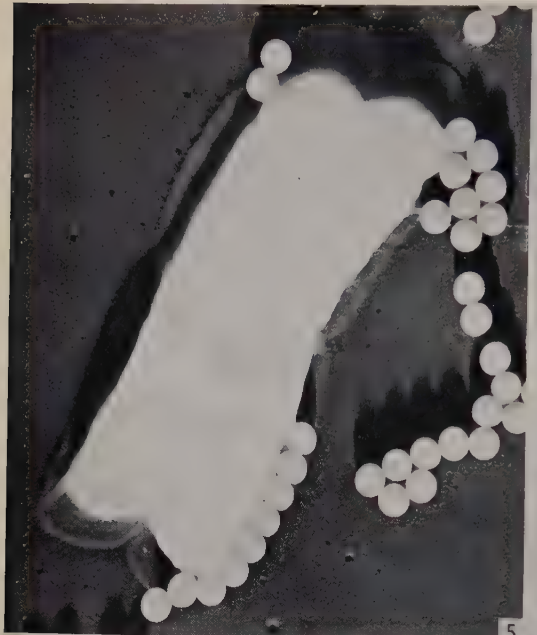


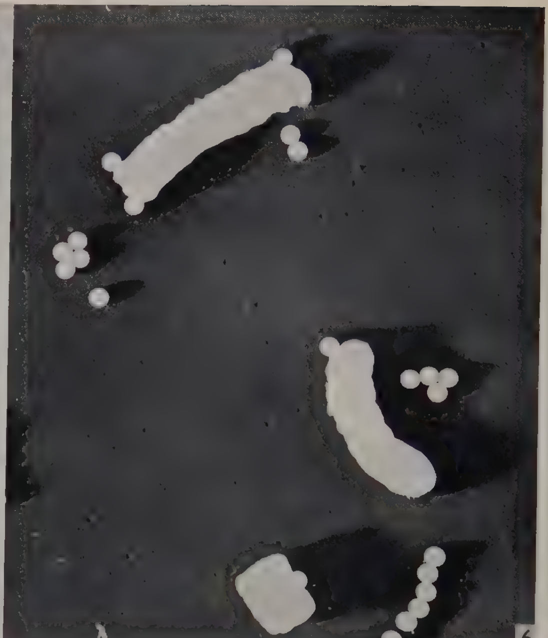


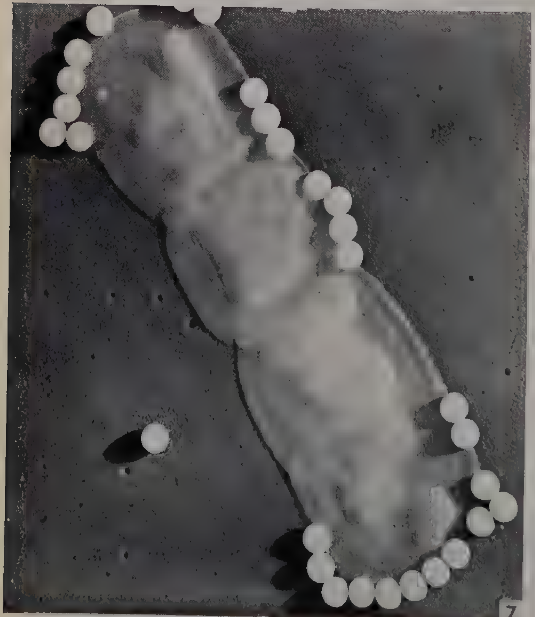


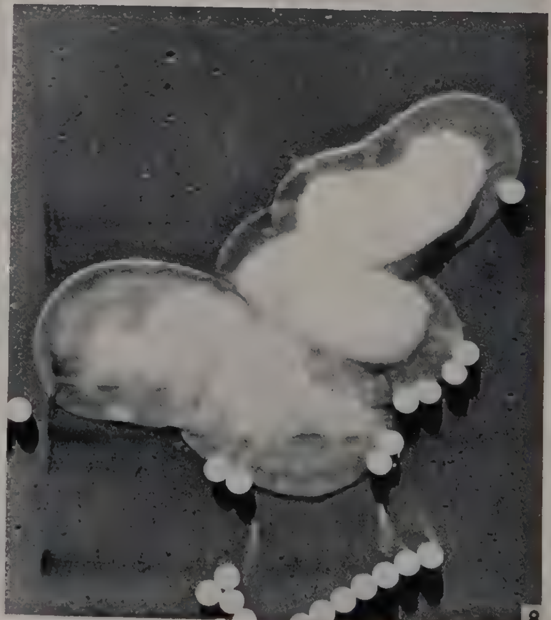


M. R. J. SALTON—ENZYMIC LYSIS OF BACTERIA. PLATE 1


5


6


7


8

M. R. J. Salton—Enzymic lysis of bacteria. Plate 2

WEBB, M. (1948). The action of lysozyme on heat-killed Gram-positive micro-organisms. *J. gen. Microbiol.* 2, 260.

WEIDEL, W. (1951). Über die Zellmembran von *Escherichia coli* B. *Z. Naturf.* 6*b*, 251.

WELSCH, M. (1947). *Phénomènes d'antibiose chez les Actinomycètes.* Gembloux: Imprimerie J. Duculet.

WELSHIMER, H. J. & ROBINOW, C. F. (1949). The lysis of *Bacillus megatherium* by lysozyme. *J. Bact.* 57, 489.

EXPLANATION OF PLATES

All photographs are electron micrographs of preparations shadowed with uranium. The electron-dense spheres are polystyrene latex indicator particles.

PLATE 1

Fig. 1. Residue from suspension of heat-killed *Bact. coli* lysed with crude trypsin (1 mg./ml.) for 2 hr. at 37°. ×15,000.

Fig. 2. Residue from suspension of heat-killed *Ps. fluorescens* lysed with crude trypsin (1 mg./ml.) for 2 hr. at 37°. ×15,000.

Fig. 3. *B. megaterium* cell-wall preparation. ×12,000.

Fig. 4. Heat-killed *B. megaterium* cell. ×13,000.

PLATE 2

Fig. 5. Heat-killed *B. megaterium* cells. ×17,000.

Fig. 6. Heated *B. megaterium* cells treated with crystalline lysozyme (100 μg./ml.) for 60 min. at 37°. Note the complete removal of the cell wall by enzymic digestion. ×10,000.

Figs. 7, 8. Showing the appearance of heated cells of *B. megaterium* after incubation with crude trypsin (1 mg./ml.) for 2 hr. at 37°. Compare with heated cells in figs. 4 and 5. Fig. 7, ×16,000; fig. 8, ×13,000.

(*Received* 22 *June* 1953)

Lowbury, E. J. L. & Hood, A. M. (1953). *J. gen. Microbiol.* 9, 524–535.

The Acquired Resistance of *Staphylococcus aureus* to Bacteriophage

By E. J. L. LOWBURY and A. M. HOOD

Medical Research Council Industrial Injuries and Burns Research Unit, Birmingham Accident Hospital

SUMMARY: To test the capacity of different staphylococci to acquire resistance to bacteriophage *in vitro*, twenty phages were grown on solid medium and in fluid medium with their propagating strains of *Staphylococcus aureus*, different phage types and patterns being represented.

Resistant secondary growth never appeared among staphylococci of the 3A group and often appeared among members of the 6/47 group. Some members of the 29/52 group acquired resistance readily, and others never became resistant. Cross-resistance to other phages was commonly acquired. Secondary growth was shown to be resistant or sensitive to the phage, or to yield a mixture of resistant and sensitive colonies.

With the exception of propagating strain PS 69, all staphylococci which became resistant to phage acquired lysogenicity for the sensitive parent strain. All but one of the staphylococci which had acquired resistance to a phage appeared to be capable of absorbing that phage. A substance which caused non-specific inhibition of phage lysis on agar medium was present in phage lysates of a staphylococcus that had shown sensitive secondary growth. These results are discussed with reference to phage typing and to the possibilities of therapy by phage.

During a trial of bacteriophage as a local prophylactic agent against *Staphylococcus aureus* in burns, swabs from the burns were found to contain an increasing proportion of non-typable staphylococci and of strains with a diminished sensitivity to a phage included in the prophylactic cream (Lowbury & Hood, unpublished). Such a result might be expected if staphylococci on burns were acquiring resistance to the phage. Phage-resistant secondary growth of *Staph. aureus* in fluid cultures and on agar cultures has been described (Bruynoghe & Maisin, 1921; Rountree, 1947; Williams Smith, 1948; Nicolle & Conge, 1949). To assess the therapeutic potentialities of phage it would be desirable to know the capacity of a wide range of organisms to acquire resistance to it. In the experiments described below we examined the propagating strains of twenty different phages, (including representatives of the four groups; Williams & Rippon, 1952), for the production of secondary growth in fluid and on solid media and the sensitivity of this secondary growth to phage. All strains were examined for acquired lysogenicity to the sensitive parent strains, and for cross-resistance to other phages. Resistant strains were also tested for ability to absorb the phage to which they had become resistant.

MATERIALS AND METHODS

Phages and strains of Staphylococcus aureus. Filtrates were obtained as described by Williams & Rippon (1952) from the following phages: 3A, 3B, 3C, 55, 52, 52A, 42E, 47, 70, 54, 7, 75, 29, 31, 6, 53, 42D, 69, 44 and 75B. The strains of *Staph. aureus* were those used for the routine propagation of the phages, and were designated by the same numbers with the prefix PS (propagating strain). The same propagating strain (PS 31/44) was used for phages 31 and 44. For the experiments described below, filtrates with a titre of not less than 1/1000 were used, the end-point chosen being semiconfluent lysis.

Culture media. Nutrient broth was used for fluid culture. The agar culture medium used for propagation and for typing was a modification of the one previously used by Hood (1953), and consisted of the following ingredients: Yeastrel, 3 g.; peptone (Allen & Hanburys Eupepton no. 2), 5 g.; sodium chloride, 3 g.; sodium dihydrogen phosphate, 3 g.; shred agar, 12·5 g.; water, 1000 ml. This mixture was steamed, adjusted to pH 8·2–8·4, filtered, re-adjusted to pH 7·4–7·6, and autoclaved at 15 lb./sq.in. for 15 min. Before use, 50 ml. horse serum were added to the melted agar; this enrichment was found to improve the propagation of most of the phages and the appearance of lysis.

Propagation of phage and cultivation of secondary growth in fluid culture medium. Two ml. of undiluted filtrate of each phage were mixed with an equal volume of an overnight broth culture of its propagating strain of *Staph. aureus*. After 3 days incubation at 37° (and again after 6 days when phage-resistant growth was not found after 3 days) these tubes were examined for turbidity and subcultured to blood agar plates. Ten colonies from the growth appearing on each of these plates after incubation were subcultured in nutrient broth and typed with diluted and with undiluted phage filtrates by a modification of the method of Wilson & Atkinson (see Hood, 1953).

Propagation of phage and cultivation of secondary growth on solid culture medium. On Yeastrel serum agar plates small areas were inoculated by wire loop with 18 hr. broth cultures of the nineteen strains of *Staph. aureus*. When these inocula had dried, loopfuls of undiluted phage filtrate were inoculated on their respective propagating strains of staphylococcus. The plates were incubated overnight at 37° and examined for lysis and secondary growth. Whether secondary growth was present or not, a sterile loop was rubbed over the lysed area and inoculated into nutrient broth. After 4–5 hr. incubation at 37°, the broth tubes were subcultured to blood agar plates, which were incubated at 37° for 24 hr. Ten colonies of *Staph. aureus* were then picked from each plate to nutrient broth, the growth in which was typed as described above.

Tests for lysogenicity of secondary growth. Ten colonies obtained by subculture from secondary growth of all strains, whether sensitive or resistant, were inoculated into nutrient broth. At least three subcultures were made in broth in order to eliminate transfer of phage from the original suspensions. Tubes of the last subculture were incubated at 37° for 4–5 hr., and the contents were centrifuged for 10 min. at 3000 r.p.m. The supernatant fluids were spotted on plates of Yeastrel serum agar, on which broth cultures of the sensitive parent staphylococci had been flooded and allowed to dry. These plates were incubated at 30° overnight, then examined for lysis. Phages from resistant cultures shown to be lysogenic for the sensitive parent organisms were propagated in the usual way, and the lytic spectra were found (see Williams & Rippon, 1952). Similar tests of lysogenicity were made on ten colonies of subcultures from residual growth in broth cultures of staphylococci with phage.

Absorption of phage by staphylococci which acquired resistance. To determine whether staphylococci which have acquired resistance to a phage will absorb the same phage, 1 drop of undiluted phage filtrate was mixed with 5 ml. of a 24 hr. broth culture of *Staph. aureus.* Immediately 0·1 ml. of this mixture was transferred to 0·9 ml. broth and the diluted suspension was centrifuged. The phage particle concentration in the supernatant was estimated from plaque counts on plates of the propagating strain spotted with 0·02 ml. amounts of tenfold dilutions of the phage suspension. The approximate number of phage particles/ml. supernatant was also obtained after incubation of the mixture for 1 and 6 hr. at 37°. Phage particle counts were also made on control phage suspensions which were incubated for 6 hr. without staphylococci.

RESULTS

Secondary growth on solid and in fluid media

From Table 1 it can be seen that visible secondary growth appeared in the zone of lysis of all cultures on agar medium, with the exception of PS3A, PS3B, PS53 and PS42D; the secondary growth of PS31/44 and PS69 was poor. In fluid culture complete lysis was not found in any of the mixtures of staphylococci and phage. The organisms in this turbid medium might therefore consist either of secondary growth or of a mixture of residual organisms not attacked by the phage and secondary growth which occurred after the addition of phage.

Phage sensitivity of secondary growth

The results are summarized in Tables 1 and 2. In the experiments with agar medium, residual organisms from the zones of lysis of the four staphylococcal strains showing no visible secondary growth were detected on subculture in broth, and tested in the same manner as subcultures of secondary growth from other staphylococci.

Secondary or residual growth of PS3A, PS3B, PS3C, PS55, PS52, PS52A and PS42E from propagation either on solid or in fluid medium was never found to be resistant to the corresponding phages. On the other hand, secondary or residual growth of PS7 and PS75 from solid or fluid medium was always found to be resistant to phages 7 and 75 respectively. PS53 showed resistant secondary growth in broth, and no growth from subculture of zones of lysis on agar. The other staphylococci yielded a mixture of sensitive and resistant colonies in the subcultures from secondary or residual growth. The proportions of sensitive and resistant colonies obtained from solid and fluid media varied widely. PS31/44 with phage 44 gave entirely different results by the two methods; subcultures from zones of lysis on agar yielded ten colonies resistant to phage 44, while forty colonies from secondary or residual growth of this organism in broth were sensitive to the phage. PS75B, in contrast, gave 10/10 sensitive colonies from secondary growth on agar and 9/10 resistant colonies from broth.

Table 1. *Changes in phage sensitivity after incubation of phages with their propagating strains of* Staphylococcus aureus

Parent strains of *Staph. aureus*		Phages with which parent strains were incubated	Phage type or pattern of resistant secondary or residual growth on agar or in broth (at RTD)	Sensitivity (S) or resistance (R) to appropriate phage of ten colonies from secondary or residual growth						Lysogenicity of secondary growth for parent strain
				On agar plates			In broth			
No.	Phage type or pattern (at RTD)*			Visible secondary growth	S	R	Visible growth	S	R	
PS3A	3A	3A	.	None	10†	0	+	10	0	Nil
PS3B	3B	3B	.	None	10†	0	+	10	0	Nil
PS3C	3C	3C	.	+	10	0	+	10	0	Nil
PS55	55	55	.	+	10	0	+	10	0	Nil
PS52	52	52	.	+	10	0	+	10	0	Nil
PS52A	52A	52A	.	+	10	0	+	10	0	Nil
PS42E	42E	42E	.	+	10	0	+	10	0	Nil
PS47	47/75/75B/77	47	75B	+	2	8	+	1	9	+
PS70	70/75/76/77	70	76/77	+	6	4	+	0	10	+
PS54	47/53/54/75/75A/75B+	54	75B	+	1	9	+	4	6	+
PS7	7	7	N.T.	+	0	10	+	0	10	+
PS75	75/76/77	75	76/77	+	0	10	+	0	10	+
PS29	29	29	N.T.	+	4	6	+	2	8	+
PS31/44	31/44+	31	N.T.‡	+	0	10	+	1	9	+
PS6	6/53A/54/75+	6	75+	+	4	6	+	1	9	+
PS53	53/75A/75B+	53	N.T.	None	No growth		+	0	10	+
PS42D	42D	42D	N.T.	None	10†	0	+	3	7	+
PS69	69	69	N.T.	±	0	10	+	2	8	Nil
PS31/44	31/44+	44	31+	±	0	10	+	10	0	+
PS75B	75B	75B	N.T.	+	10	0	+	1	9	+

* Routine test dilution.

† Although visible secondary growth was absent, growth appeared on broth subculture from the lysed areas.

‡ Weak reaction with phages other than 31/44 persisted.

Table 2. *Loss of sensitivity to undiluted phage filtrates after incubation of staphylococci with phages*

Strain of *Staph. aureus*	Phage incubated with the staphylococcus	Phage pattern of parent strain	Phage pattern of strain which acquired resistance
PS47	47	**47**/53/54/75/75A/75B/76/77/70/**47B**/**47C**/31B	53/75/75A/75B/76/77/70/54/31B
PS70	70	**70**/**75**/**75A**/**75B**/76/77/**47**/**47B**/**47C**/53/**54**	53/76/77
PS54	54	**47**/53/**54**/75/75A/75B/76/77/70/**47B**/**47C**	53/70/75/75A/75B/76/77
PS7	7	6/**7**/42B/42E/47B/**47C**/53/54/57/**47**/**47D**/**75**/**75A**/**76**	6/42B/42E/47B/53/54/57
PS75	75	**47**/**75**/**53**/**75A**/**75B**/**70**/76/77	76/77
PS29	29	**29**/**31**	N.T.
PS31/44	31	7/47/47B/47C/47D/53/53A/54/**70**/75/75A/75B/76/77/**78**/**29**/**29A**/**31**/**31A**/**52**/**52A**/**69**/42B/**42E**/**44**	7/42B/47/47B/47C/54/47D/75/75A/75B/76/77
PS6	6	**6**/47/**47B**/47C/53/**53A**/54/75/75A/75B/**47C**/76/77/**42C**	47/53/54/75/75A/75B/76/47C/77
PS53	53	**53**/**75**/76/77	76/77
PS42D	42D	**42D**	N.T.
PS69	69	**69**	N.T.
PS31/44	44	7/47/47B/47C/47D/53/53A/54/70/75/75A/75B/76/77/78/29/**29A**/31/31A/52/**52A**/69/42B/42E/**44**	7/47/47B/47C/47D/53/53A/54/70/75/75A/75B/76/77/78/29/31/31A/52/69/42B/42E
PS75B	75B	47/**75**/**75B**	47

Note. The numbers in heavy type represent phages that lysed the parent strain but not the strain which acquired resistance.

Stability of acquired resistance

After twenty serial subcultures, alternately from colonies on blood agar and from broth, PS75 which had acquired resistance to phage showed no signs of a reversion to sensitivity. Acquired resistance and lysogenicity were also found to persist after at least six serial subcultures of the other strains described above.

Cross-resistance of staphylococci to phages other than those propagated upon them

The phage type of sensitive secondary growth, tested with filtrates at the routine test dilution (RTD) or undiluted, was the same as that of the parent strain. The phage type or pattern of resistant secondary growth, however, was shown to differ from that of the parent strain by the loss of sensitivity to the phage used in attempts to induce resistant secondary growth, and sometimes also to other phages (see Table 2). For example, *Staph. aureus* PS75 incubated with phage 75 lost its sensitivity to undiluted filtrates of phages 47, 75, 53, 75A, 75B and 70, retaining sensitivity to phages 76 and 77 only. Resistant secondary growth on agar and in broth showed the same changes in phage pattern. In no instance did a staphylococcus remain sensitive to the phage propagated upon it and acquire resistance to other phages. There was some tendency for resistance to be acquired towards closely related phages (e.g. 75 and 75A; 47, 47B and 47C).

Derivation of non-typable from typable staphylococci

Resistant secondary growth from strains PS29, PS42D, PS69, PS7, PS31/44, PS53, and PS75B was non-typable by phages at the RTD; the first three of these were also non-typable by undiluted phages.

Lysogenicity of secondary growth of Staphylococcus aureus

With the exception of PS69, all the strains of *Staph. aureus* which had acquired resistance to the propagated phage were found to be lysogenic towards the parent strains. The lytic spectra of the phages propagated from the resistant organisms on the parent strains were identical with those of the phages against which the organism had been made resistant (e.g. the resistant secondary growth of PS75 carried a phage with lytic spectrum the same as that of phage 75). Sensitive secondary growth was not found to be lysogenic for the parent strain.

Absorption of phages by strains which acquired resistance to them

The number of phage particles remaining in suspension after 1 hr. and after 6 hr. incubation with the resistant staphylococci are shown in Table 3. There was considerable variation in the degree of absorption, ranging from complete absorption of phage 69 to an apparent failure of absorption of phage 70. The degree of absorption was not related to the concentration of the

Table 3. *Absorption of phage by phage-resistant staphylococci*

Staphylococcus	Phage	Phage particles in supernatant, Particles/ml: Without added phage	Phage particles in supernatant, Particles/ml: Immediately after addition of phage	Percentage of phage particles persisting in supernatant of phage-staphylococcal mixture at 37° after: 1 hr.	Percentage of phage particles persisting in supernatant of phage-staphylococcal mixture at 37° after: 6 hr.	Phage particles in broth suspension at 37° after, Particles/ml.: 0 hr.	Phage particles in broth suspension at 37° after, Particles/ml.: 6 hr.	Percentage after 6 hr.
PS6	6	$10^4 \times 55$	$10^7 \times 3$	3·0	2·7	$10^6 \times 1{\cdot}1$	$10^6 \times 1{\cdot}4$	127
PS7	7	500	$10^6 \times 4$	0·62	0·18	$10^6 \times 45$	$10^6 \times 9$	20
PS53	53	$10^3 \times 9$	$10^4 \times 25$	12·0	0·52	$10^6 \times 3$	$10^6 \times 2$	66
PS42D	42D	50	$10^5 \times 5$	80·0	20·0	$10^3 \times 6$	$10^3 \times 3{\cdot}5$	58
PS70	70	$10^4 \times 1$	$10^5 \times 15$	67·0	47·0	$10^6 \times 1{\cdot}5$	$10^6 \times 0{\cdot}5$	33
PS29	29	$10^3 \times 3$	$10^6 \times 1$	1·0	0·8	$10^6 \times 3$	$10^6 \times 1$	30
PS69	69	0	$10^4 \times 6$	0	0	$10^3 \times 4$	$10^3 \times 6$	150
PS31/44	31	$10^6 \times 52$	$10^8 \times 2$	110	0·25	$10^6 \times 3$	$10^6 \times 1$	33
PS75B	75B	200	$10^6 \times 75$	2·6	0·001	$10^6 \times 3{\cdot}5$	$10^6 \times 2{\cdot}5$	71
PS47	47	$10^5 \times 1{\cdot}5$	$10^5 \times 2$	50	30	$10^6 \times 1$	$10^6 \times 1$	100
PS54	54	$10^5 \times 25$	$10^7 \times 5$	30	0·024	$10^6 \times 5{\cdot}5$	$10^6 \times 3{\cdot}7$	62
PS75	75	$10^4 \times 6$	$10^6 \times 4$	32·5	3·7	$10^6 \times 15$	$10^6 \times 15$	100
PS31/44	44	$10^5 \times 2$	$10^6 \times 1$	20	15	$10^4 \times 2$	$10^4 \times 2{\cdot}5$	125

phage in the lysogenic bacterial suspension before the addition of phage. Nevertheless, the only staphylococcus which was not lysogenic for the sensitive parent strain (PS 69) effected the most complete absorption of phage.

The emergence of secondary phage-sensitive organisms

Phage inhibitors in extracts of staphylococci have been described by Rakieten, Rakieten & Doff (1936) and by Freeman (1937). It seemed to us likely that the appearance of secondary phage-sensitive organisms in our cultures might be due to such inhibitors appearing in the cultures. To test this hypothesis we made the following experiments. Phages 75B, 69 and 44 and their propagating strains of *Staph. aureus* were chosen because they presented three different patterns in the phage sensitivity of secondary or residual growth (see Table 1). Agar plate propagations of the three phages were set up in the usual way. After 6 hr. incubation at 37°, the plates were placed in the refrigerator freezing unit and removed after 18 hr. to thaw at room temperature. The fluid pipetted from the thawed plates was centrifuged and filtered through a Seitz E.K. pad. Broth propagations were carried out by incubating equal volumes of undiluted phage suspension and broth culture of the propagating strain at 37°. After 3 days incubation the suspensions were centrifuged and filtered. A part of each filtrate was heated at 52° for 1 hr. Control filtrates from staphylococcal cultures without added phage were also obtained. These filtrates were tested for the presence of inhibitors of phage lysis by 'spotting' them on plates newly inoculated as for phage propagation but with higher concentrations (usually × 10) of phage. All three phages were spotted on each propagation plate; the plates were then incubated overnight at 37° and examined. The principal findings are shown in Table 4 and in Pl. 1, figs. 1–3. On each plate there was diffuse lysis with a fine layer of

Table 4. *The inhibition of phage lysis on agar by application of additional phage filtrate*

Phage and *Staph. aureus* spread on test plate	Filtrate 'spotted' on test plate	Presence of zone of heavier growth where filtrate was 'spotted', and its sensitivity to appropriate phage	
		From agar propagation	From broth propagation
75B+PS75B	Phage 75B	+++ (sensitive)	+ (sensitive)
	PS75B	0	0
	Phage 44	0	0
	Phage 69	0	0
44+PS31/44	Phage 44	0	+ (sensitive)
	PS31/44	0	0
	Page 69	0	0
	Phage 75B	+++ (sensitive)	0
69+PS69	Phage 69	+ (resistant)	0
	PS69	0	0
	Phage 44	0	0
	Phage 75B	+++ (sensitive)	± (sensitive)

secondary growth of the propagating strains, PS75B, showing rather more secondary growth than PS69 and PS31/44. In addition, the following appearances were observed:

At the site where filtrates from agar propagation of phage were 'spotted'. On all the plates disks of heavy growth appeared where phage 75B filtrate was spotted. A slight accentuation of growth occurred where phage 69 filtrate was spotted on the plate with phage 69 and PS69 (69+/PS69). No similar increase of growth occurred where this phage was spotted on plates of 44+PS31/44 and 75B+/PS75B. Phage 44 filtrate caused increased lysis where it was spotted on plate 44+PS31/44, but in other respects resembled phage 69 filtrate. Ten colonies subcultured from the disks of heavy growth in the areas spotted with phage 75B filtrate were found to be sensitive to their corresponding phages. The slightly accentuated growth of PS69 in the area spotted with phage 69 was resistant to this phage. The diffuse secondary growth on the propagation plate of phage 75B was sensitive, but that on the propagation plates for phages 44 and 69 was resistant to the propagated phage.

At the site where filtrates from broth were 'spotted'. Some increase in staphylococcal growth was found on the propagation plate for phage 44 where filtrate from the broth propagation of phage 44 had been 'spotted'. Subcultures from this growth were sensitive to phage 44. A similar increase in growth occurred where phage 75B broth propagation was 'spotted' on a propagation plate of 75B. Neither of these filtrates caused increased growth on plates spread with the other staphylococci and phages. No increase of growth appeared on any plate at the site of application of phage 69 broth filtrate.

Action of the phage inhibitor on twelve different phages. Filtrate from agar plate propagation of phage 75B was applied to areas on an agar plate previously inoculated with propagating strains and phages (at RTD) 6, 7, 47, 47A, 47C, 31A, 31B, 53, 53A, 71, 76 and 75B. A heavy growth of staphylococci appeared in all these areas; by contrast a control plate with no added phage 75B showed confluent lysis with little or no growth of staphylococci. These results show that the phage inhibitor is not specific for the staphylococcus and phage from which it was obtained.

Local application of other filtrates. Lysis was not inhibited by filtrates from the control agar or broth cultures of staphylococci without phage or from heated filtrates from agar or broth propagation of phage. Stock phages 75B, 69 and 44 used for routine typing of staphylococci gave the same results as the special agar plate propagations used in the experiment.

Interpretation of results. Lysis by several different phages was inhibited in the presence of filtrates from agar propagation of phage 75B, and smaller amounts of inhibitor could also be detected in a filtrate of a broth propagation of this phage. No such inhibitor was present in the filtrates from agar propagations of phages 44 and 69. A small amount of the inhibitor was, however, present in a filtrate from broth propagation of phage 44. The titre of phage in this filtrate and that from broth propagation of phage 75B was much lower than that obtained from the agar plate propagation. The increased growth of

PS 69 at the site of application of phage 69 filtrate was resistant to the phage, unlike that which occurred where 75 B was applied; it appeared therefore to be caused not by an inhibitor of phage lysis but by some other mechanism.

DISCUSSION

In our experiments different phage types of *Staph. aureus* differed widely in their capacity to acquire resistance to the phages propagated on them. In particular, no evidence of acquired resistance was shown by any of the staphylococci in the phage group 3 A, whereas staphylococci of the 6/47 phage group acquired resistance very readily. The behaviour of the groups in this respect seems analogous to their behaviour in relation to penicillin and to aureomycin. Penicillin-resistant *Staph. aureus* strains isolated from patients in hospital are most commonly found to belong to the 6/47 group (Barber & Whitehead, 1949; Lowbury, Topley & Hood, 1952). In this M.R.C. Unit, during a period when both penicillin and aureomycin were used routinely, the great majority of staphylococci showed certain patterns of the 6/47 group and were resistant to penicillin and many also to aureomycin. Most of the small number of staphylococci in the 3 A group which were isolated from burns during this time were sensitive to penicillin and to aureomycin. Barber & Whitehead (1949) suggest the possibility that strains of the 6/47 group are genetically less stable than those of the other phage groups. This suggestion gains support from our finding that staphylococci in the 6/47 group have a greater capacity than other staphylococci to acquire resistance to phage.

D'Herelle (1922) recognized both lysogenic and non-lysogenic strains among organisms which had acquired resistance to phage. In our study, all but one of the staphylococci (PS 69) were found to have become lysogenic for the parent strain when they became resistant to the phage. Absorption of phage was shown in varying measure by all but one of the staphylococci which had acquired resistance; in this respect their behaviour is typical of staphylococci, which are known to absorb phages that do not lyse them (Burnet & Lush, 1935; Rountree, 1947; Hood, 1953). Our result, however, does not corroborate the assumption of Delbrück (1946) that 'resistance is primarily caused by a loss of the capacity for adsorbing a specific virus'.

The secondary growth which appears in the zone of lysis may be either resistant or sensitive to the phage, or consist of a mixture of sensitive and resistant organisms. The appearance of secondary sensitive growth could be explained if inhibitors of phage lysis were liberated in the medium on which staphylococci were lysed by phage. Rakieten *et al.*(1936) and Freeman (1937) described phage inhibitors in extracts of staphylococci. Our experiments showed the presence of such an agent in filtrates from the agar propagation of phage 75 B on its propagating strain of *Staph. aureus*, an organism which had shown secondary sensitive growth in zones of lysis. Similar filtrates of phages 44 and 69, however, were not found to contain such an inhibitor of phage lysis; this observation was consistent with the absence of sensitive secondary growth in zones of lysis produced by phages 44 and 69 on their propagating strains of *Staph. aureus*. Filtrate from the plate propagation of phage 75B was found to inhibit phage lysis of a number of different staphylococci, including that of PS69 by phage 69 and of PS31/44 by phage 44. The inhibitor is therefore not specific in its action. It differs from the phage inactivator described by Rakieten *et al.* in being thermolabile. Some increased growth of PS69 occurred at the site of application of a filtrate of phage 69; this growth, unlike that stimulated by applying a filtrate of phage 75B, was resistant to phage 69, and must be considered to have appeared through some other mechanism, e.g. the quicker emergence of resistant organisms where phage particles are more abundant. Discrepancies between the phage sensitivity of secondary or residual growth on agar and in broth are consistent with the results of these experiments, e.g. evidence of an inhibitor of phage lysis could be found in filtrates from broth but not from agar propagation of phage 44; filtrates from agar propagation of phage 75B, on the other hand, showed evidence of a greater concentration of inhibitor than could be found in filtrate from broth propagation. Further study is being made on this phenomenon.

Our results, like those of Williams Smith (1948), show that considerable difference in the phage pattern of staphylococci may occur as the result of acquired resistance. Staphylococci which are lysed by a 'pattern' of phages may become resistant to several of them after growing in the presence of one phage; they may also become non-typable. The possibility that such changes may occur in the environment (Williams Smith, 1948) shows the severe limitation of phage pattern as a descriptive label for staphylococci.

Successful phage therapy might be expected in infection by staphylococci of the phage group 3A, or in other staphylococci which do not readily acquire resistance to the phage. But since these are probably the organisms that also more readily retain their sensitivity to the antibiotics, phages are unlikely to contribute much to the therapy or prophylaxis of staphylococcal infections.

REFERENCES

Barber, M. & Whitehead, J. E. M. (1949). Bacteriophage types in penicillin-resistant staphylococcal infection. *Brit. med. J.* ii, 565.

Bruynoghe, R. & Maisin, J. (1921). Le principe bactériophage du staphylococque. *C.R. Soc. Biol., Paris*, **85**, 1118.

Burnet, F. M. & Lush, D. (1935). The staphylococcal bacteriophages. *J. Path Bact.* **40**, 455.

Delbrück, M. (1946). Bacterial viruses or bacteriophages. *Biol. Rev.* **21**, 30.

Freeman, M. (1937). The phage inactivating agent in extracts of *Staphylococcus aureus*. *Aust. J. exp. Biol. med. Sci.* **15**, 221.

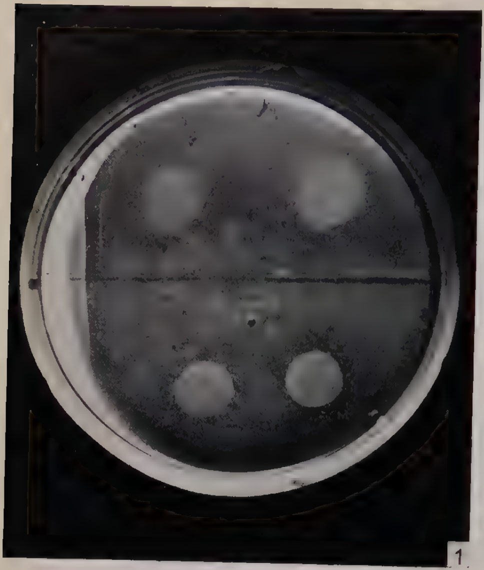
d'Herelle, F. (1922). *The Bacteriophage: its Role in Immunity.* (Quoted in Hadley, P. (1928), *J. infect. Dis.* **42**, 263.)

Hood, A. M. (1953). Phage typing of *Staphylococcus aureus*. *J. Hyg., Camb.* **51**, 1.

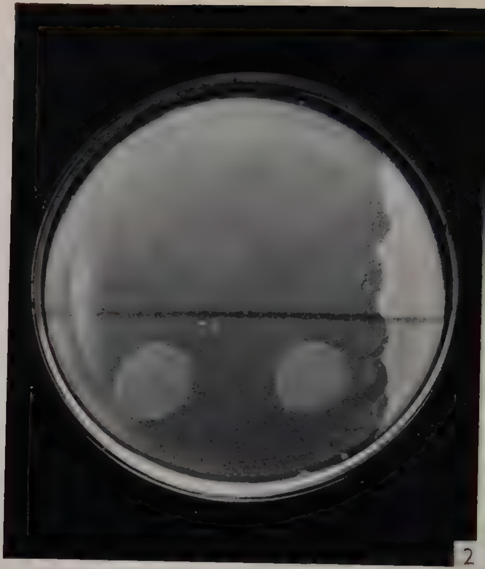
Lowbury, E. J. L., Topley, E. & Hood, A. M. (1952). Chemotherapy for *Staphylococcus aureus* in burns. *Lancet*, i, 1036.

Nicolle, P. & Conge, G. (1949). Le théorie des mutants spontanés: est elle applicable a tous les cas de cultures secondaires après bactériophagie? *Ann. Inst. Pasteur*, **76**, 381.

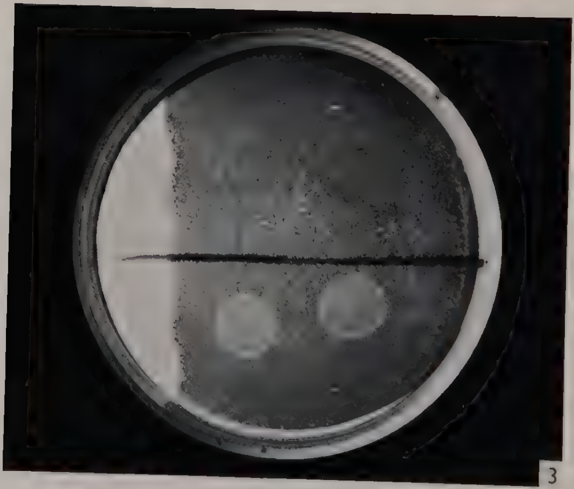
Rakieten, M. L., Rakieten, T. L. & Doff, S. (1936). Absorption of staphylococcal phages. *J. Bact.* **32**, 505.



1



2



3

E. J. L. LOWBURY AND A. M. HOOD—ACQUIRED RESISTANCE TO BACTERIOPHAGE. PLATE 1

ROUNTREE, P. (1947). Bacteriophage absorption by staphylococci. *Aust. J. exp. Biol. med. Sci.* 25, 203.

WILLIAMS, R. E. O. & RIPPON, J. (1952). Bacteriophage typing of *Staphylococcus aureus*. *J. Hyg., Camb.* 50, 320.

WILLIAMS SMITH, H. (1948). Investigations on the typing of staphylococci by means of bacteriophage. *J. Hyg., Camb.* 46, 74, 82.

EXPLANATION OF PLATE

Fig. 1. Propagation plate of phage 69 on PS 69. The two zones of heavier growth in the upper half of the plate are at the site of application of phage 69 filtrate, and those in the lower half are at the sites of application of phage 75 B filtrate, both filtrates having been applied before incubation of the plate.

Fig. 2. Propagation plate of phage 75 B on PS 75 B. In the lower half of the plate are two zones of heavier growth where phage 75 B filtrate was 'spotted' before incubation of the plate. No zones of heavier growth appeared in the upper half of the plate, where phage 69 filtrate was applied before the plate was incubated.

Fig. 3. Propagation plate of phage 44 on PS 31/44. As in figs. 1 and 2, zones of heavier growth appeared in the lower half of the plate where phage 75 B filtrate was applied before incubation. In the upper half of the plate are two areas of increased lysis at the site of application of phage 44 filtrate before incubation.

(*Received* 22 *June* 1953)

Nimmo-Smith, R. H. & Brown, D. J. (1953). *J. gen. Microbiol.* 9, 536–544.

Some Effects of 2-Deaminopteroylglutamic Acid upon Bacterial Growth

By R. H. NIMMO-SMITH
Microbiology Unit, Department of Biochemistry, Oxford
AND D. J. BROWN
Department of Medical Chemistry, Australian National University

SUMMARY: Four analogues of pteroic acid and one of pteroylglutamic acid were tested for their ability to stimulate growth of *Streptococcus faecalis* R in the absence of pteroic acid or to inhibit growth in its presence. The four analogues of pteroic acid were inactive in either respect, but 2-deaminopteroylglutamic acid was inhibitory. Inhibition of the organism was most marked in the presence of pteroic acid, and became relatively more efficient as the concentrations of inhibitor and growth factor were increased. Inhibition was much less marked in the presence of rhizopterin or Leucovorin, the relative efficiency decreasing in comparable conditions. Pteroylglutamic acid had an intermediate position and showed a competitive relationship.

Lactobacillus casei was similarly inhibited when growth was supported by either pteroylglutamic acid or Leucovorin, but was less pronounced in the presence of the latter. On the other hand, the analogue could substitute for pteroylglutamic acid in stimulating the suboptimal growth supported by a mixture of thymine and a purine in the absence of added folic acid.

Several other organisms whose growth is independent of exogenous folic acid were unaffected by the analogue.

The synthesis of analogues and derivatives of pteroic acid and pteroylglutamic acid has been undertaken in several laboratories in order to throw light upon the functioning of the metabolically important folic acid group of substances (e.g. see reviews by Jukes, Franklin & Stokstad, 1950; Woolley, 1952). The synthesis of 4-hydroxy-6-methylpteridine (Albert, Brown & Cheeseman, 1952) made it possible for the first time to prepare analogues which lack the amino-group in the 2-position. Five compounds of this type have been synthesized (Brown, 1953), and their structures are indicated in Fig. 1.

METHODS

Organisms

Streptococcus faecalis R (American Type Culture Collection, ATCC no. 8043) was maintained by stab subculture in medium A at 37°.

Lactobacillus casei (ATCC No. 7469) was similarly maintained in medium B1.

Media

Medium A. For each 100 ml.: Marmite, 1 g.; glucose, 0·5 g.; sodium acetate trihydrate, 1 g.; agar, 1·5 g.; pH adjusted to 6·7–6·9.

*Medium B*1. For each 100 ml.: Tryptose (Difco), 1 g.; yeast extract (Difco), 0·2 g.; glucose, 1 g.; K_2HPO_4, 0·5 g.; liver extract (Oxoid, 16% w/v), 10 ml.;

$MgSO_4.7H_2O$, 20 mg.; $FeSO_4.7H_2O$, 1 mg.; $MnSO_4.4H_2O$, 1 mg; $CaCO_3$, 1·5 g.; agar, 1·5 g.

*Medium B*2. As medium B1 but without $CaCO_3$ and agar; pH 6·7–6·9.

Medium C. This was the medium of Rabinowitz & Snell (1947) with DL-cysteine (200 mg./l.) instead of L-cysteine, with pyridoxin added at 1 mg./l. and with folic acid and *p*-aminobenzoic acid left out.

Medium D. The culture medium for *L. casei* was that modification of the Roberts & Snell (1946) medium previously described (Nimmo-Smith, Lascelles & Woods, 1948).

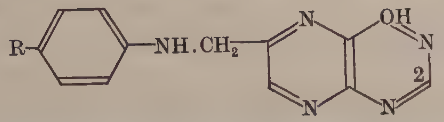


Fig. 1. The five compounds investigated were: 6-anilinomethyl-4-hydroxypteridine (R=—H); 6-*p*-anisidinomethyl-4-hydroxypteridine (R=—OCH_3); 4-hydroxy-6-*p*-sulphanilinomethylpteridine (R=—SO_3H); 6-*p*-carboxyanilinomethyl-4-hydroxypteridine (R=COOH) and 2-deaminopteroylglutamic acid

[R=—CONH.CH(COOH).$(CH_2)_2$.COOH].

Materials

Pteroic acid, pteroylglutamic acid and Leucovorin (5-formyl-5 : 6 : 7 : 8-tetrahydropteroyl-L-glutamic acid; 'folinic acid') were synthetic samples generously supplied by Lederle Laboratories Division. Rhizopterin (N^{10}-formylpteroic acid) was kindly given by the Merck Co., Inc. Stock solutions of each at 10^{-3}M in 10^{-2}M-$NaHCO_3$ were stored in the ice-chest and diluted when needed.

Stock solutions (10^{-3}M in sterile 2×10^{-2}N-NaOH) of the five analogue compounds were similarly stored.

Methods of cultivation

The same general precautions were observed as in conducting folic acid assays (Nimmo-Smith, Lascelles & Woods 1948). Samples of the media (at 70% of final volume) were distributed in $5\times\frac{5}{8}$ in. hard-glass test-tubes. Suitable additions of diluted growth factor were made and, where necessary, distilled water was added so that subsequent additions of analogue solution and inoculum brought the contents of each tube to the same final volume.

The tubes were autoclaved for 10 min. at 10 lb./sq.in. This treatment, at pH 6·8, causes no detectable destruction of even the relatively unstable Leucovorin (unpublished observation by Dr June Lascelles). After autoclaving, different concentrations of the analogues to be tested were added and the tubes inoculated.

Strep. faecalis was grown for 22–24 hr. at 37° in a final volume of 5·0 ml. medium C and an atmosphere of air. The inoculum was prepared by growing the organism for 18 hr. in medium B2 and diluting the culture 1/100 in sterile water. To each tube 0·5 ml. of the dilute suspension was added.

L. casei was grown for 24–26 hr. at 37° in a final volume of 2·0 ml. of medium D and an atmosphere 5% CO_2+95% H_2 (Nimmo-Smith *et al.* 1948).

The inoculum was 0·2 ml. of a 1/100 dilution in water of a 24 hr. culture of the organism in medium D supplemented with $1{\cdot}5 \times 10^{-8}$ M-pteroylglutamic acid. This had been inoculated with about 0·02 ml. of a 16 hr. culture in medium B2, inoculated in turn from a fresh 24 hr. stab culture in medium B1.

In both cases growth was assessed with a portable colorimeter (Evans Electroselenium, Ltd.), uninoculated medium being used to adjust to zero the reading on the instrument scale. A calibration of dry weight against colorimeter reading was made for each organism and the relationship shown to be linear within the range encountered. Half-maximal growth of *Strep. faecalis* in our conditions corresponded to an instrument reading of 14 (0·69 mg. dry wt./ml.) and of *L. casei* to a reading of 19 (0·65 mg. dry wt./ml.).

In the quantitative experiments growth determined in this way was plotted against the logarithm of the concentration of either growth factor or analogue, whichever had been varied. For comparative purposes the molar ratio between analogue and growth factor obtaining at the point of half-maximal growth (or half-maximal inhibition) was used. In most cases serial 1/5 dilutions of growth factor were added to groups of tubes. Within each group the concentration of analogue was fixed, but varied by a factor of 5 from one group to the next. Less commonly the analogue was similarly titrated in the presence of fixed concentrations of the growth factor.

As a first step the five compounds were 'screened' with *Strep. faecalis* R in media (*a*) without folic acid, and (*b*) containing a suboptimal concentration ($3{\cdot}3 \times 10^{-8}$ M) of pteroic acid. This technique allows the detection of compounds which can (i) replace folic acid, (ii) stimulate growth in the presence of low concentrations of folic acid or which are (iii) inhibitory.

RESULTS

Tested over a concentration range from 10^{-7} up to 10^{-4} M none of the compounds stimulated growth either on its own or in the presence of suboptimal pteroic acid. However, 2-deaminopteroylglutamic acid showed an interesting type of inhibition; experiments were carried out to determine the behaviour of this compound; the other four were not further investigated.

Effects of 2-deaminopteroylglutamic acid

Effect on growth of Strep. faecalis *R.* When pteroic acid was acting as source of folic acid the analogue showed itself to be a rather efficient inhibitor. The analogue/growth factor relationship was not a strictly competitive one, since the relative efficiency of the inhibitor increased markedly as the concentration of both was increased. This was shown by the amount of 2-deaminopteroylglutamic acid which had to be added to produce half-maximal inhibition in the presence of different concentrations of pteroic acid (Table 1). In Fig. 2 the logarithm of the molar ratio at half-maximal inhibition has been plotted against the logarithm of the analogue concentration. The position was further complicated by the fact that pterioc acid above about $3{\cdot}3 \times 10^{-6}$ M was itself slightly inhibitory.

In contrast, growth of the organism supported by rhizopterin was much less readily inhibited by the analogue, and the relative efficiency of the latter declined as concentrations were increased. When the concentration of the analogue was increased 25-fold the analogue : growth factor ratio at the half-maximal point rose by a factor of about 5 (Table 1).

Table 1. *Relationship between concentrations of 2-deaminopteroylglytamic acid and of various growth factors at half-maximal growth of* Streptococcus faecalis *R*

Pt. = pteroic acid; *RPt.* = rhizopterin; *PtG.* = pteroylglutamic acid; *LV.* = Leucovorin; *DPtG.* = 2-deaminopteroylglutamic acid.

Concentrations		
a DPtG. ($\times 10^{-5}$ M)	*b* Growth-factor ($\times 10^{-10}$ M)	Ratio *a* : *b*
	Pt.	
0	990	—
3·05	1,000	305
6·35	3,300	190
10·0	10,000	100
10·9	33,000	33
	RPt.	
0	15	—
4	15	26,700
20	20	100,000
100	71	141,000
	PtG.	
0	14	—
4	40	10,000
20	170	11,800
100	865	11,600
	LV.	
0	14	—
4	19	21,000
20	45	44,000
100	110	90,000

When pteroylglutamic acid was acting as source of folic acid, 2-deaminopteroylglutamic acid produced half-maximal inhibition at a molar ratio of about 1 : 10,000. The relationship between the two, at least over a 50-fold range of concentration, appeared to be a competitive one (Table 1).

Behaviour of 2-deaminopteroylglutamic acid in the presence of Leucovorin was very similar to that in the presence of rhizopterin. A 25-fold increase in concentration of the analogue was associated with roughly a five-fold increase in the analogue : growth factor ratio (Table 1).

Synthetic Leucovorin is a mixture of the +L and −L isomers and is approximately 50 % as active for *Strep. faecalis* R as is the −L isomer (Cosulich, Smith & Broquist, 1952), and less active than the natural material for this organism also (Sauberlich, 1952). On the basis that probably only the *l*L isomer is available, concentrations of Leucovorin in Tables 1 and 2 are expressed in terms of this isomer only.

A medium in which thymine and a purine take the place of folic acid will support adequately the growth of *Strep. faecalis* R (Stokes, 1944). In these conditions, with both thymine and hypoxanthine present at a concentration of 10^{-4} M, 2-deaminopteroylglutamic acid, tested over the range 10^{-7} up to 10^{-3} M, was completely without effect upon growth.

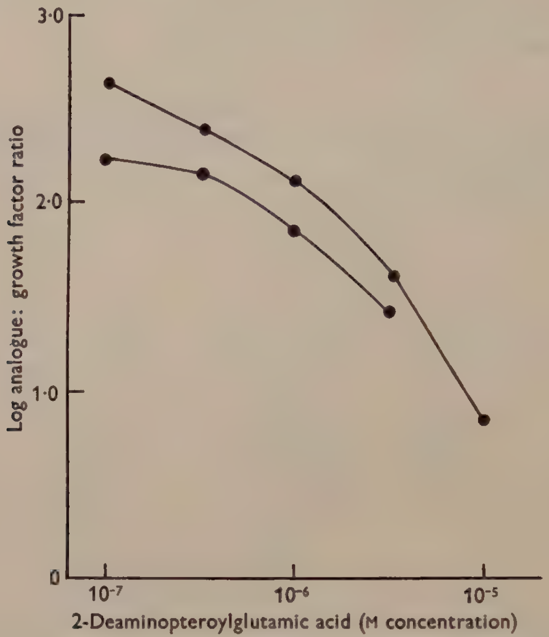


Fig. 2. Relationship between the 2-deaminopteroylglutamic acid : pteroic acid ratio at half-maximal inhibition of *Strep. faecalis* R and the absolute concentration of the analogue (two separate experiments).

Table 2. *Relationships between concentrations of* 2-*deaminopteroylglutamic acid and of growth factor at half-maximal growth of* Lactobacillus casei

Symbols (*DPtG.*, etc.) have same significance as in Table 1.

Concentration *a* *DPtG.* ($\times 10^{-5}$ M)	 *b* Growth-factor ($\times 10^{-10}$ M)	Ratio *a* : *b*
	PtG.	
0	7	—
4	24	16,700
20	44	45,500
100	144	69,000
	LV.	
0	4·8	—
4	6·2	64,000
20	11·5	174,000
100	39	255,000

Effect on growth of L. casei. Neither pteroic acid nor rhizopterin support the growth of this organism. 2-Deaminopteroylglutamic acid was inhibitory when growth of this organism was supported by pteroylglutamic acid, half-maximal inhibition being reached when the concentration of analogue was 1 to 7×10^4 times that of the growth factor.

There is some suggestion of a competitive relationship between the two but, owing to the relatively low activity and solubility of the analogue, it was not possible to test it over an adequate range of concentration. From Table 2 it can be seen that, although the concentration of 2-deaminopteroylglutamic acid was increased 25-fold the concentration of pteroylglutamic acid had only to be increased fivefold to obtain the same amount of growth.

Growth supported by Leucovorin was more resistant to inhibition (Table 2) than growth in presence of pteroylglutamic acid. Nevertheless, the progressive increase in ratio between analogue and growth factor was precisely the same in each case.

A mixture of thymine and a purine supports also the growth of *L. casei* in the absence of added folic acid. In confirmation of other workers (e.g. Stokes, 1944; Krueger & Peterson, 1945) we found that growth in these conditions, as measured turbidimetrically, was consistently about half as good as with pteroylglutamic acid. Supplementation with a relatively low concentration of the latter, however, stimulated maximal growth.

Surprisingly, 2-deaminopteroylglutamic acid possesses a similar stimulatory activity. To obtain an increase of 50% above the basal growth, it must be present at a concentration about 100 times that of the concentration of pteroylglutamic acid needed to produce the same effect (Fig. 3). 2-Deaminopteroylglutamic acid produced its maximum growth stimulatory effect at about 10^{-6} M, and stimulated growth which was always slightly suboptimal; it had no further effect on being increased to 10^{-3} M.

Effect on growth of other organisms. In addition to the above two organisms which required folic acid as nutrient the analogue was tested for its effect upon the growth of four organisms whose growth in a defined medium is independent of an exogenous supply of folic acid, namely, *Bacterium coli*, *Lactobacillus arabinosus* 17–5, *L. plantarum* 10S and *Schizosaccharomyces octosporus*. Even at 10^{-3} M neither stimulation nor inhibition of growth of any of these organisms was induced by the analogue.

DISCUSSION

The results show that 2-deaminopteroylglutamic acid is a compound which can interfere with the metabolism of folic acid in two organisms which require this vitamin as a nutrient for growth. It is interesting to note, in the case of *Strep. faecalis*, how strikingly the relationship between analogue and growth factor alters with the nature of the latter. With pteroic acid a 3·5-fold increase in the concentration of the analogue demands a 33-fold increase in growth factor concentration. Yet a 25-fold increase of analogue requires only a five-fold increase of rhizopterin to produce the same amount of growth. Both relationships are clearly not competitive, but deviate in opposite directions.

The relationship between the analogue and pteroic acid is similar to that between 4-aminopteroylglutamic acid and pteroylglutamic acid with this same organism. Oleson, Hutchings & SubbaRow (1948) found that a ninefold increase in concentration of inhibitor required a 100-fold rise in pteroylglutamic acid concentration to maintain half-maximal growth.

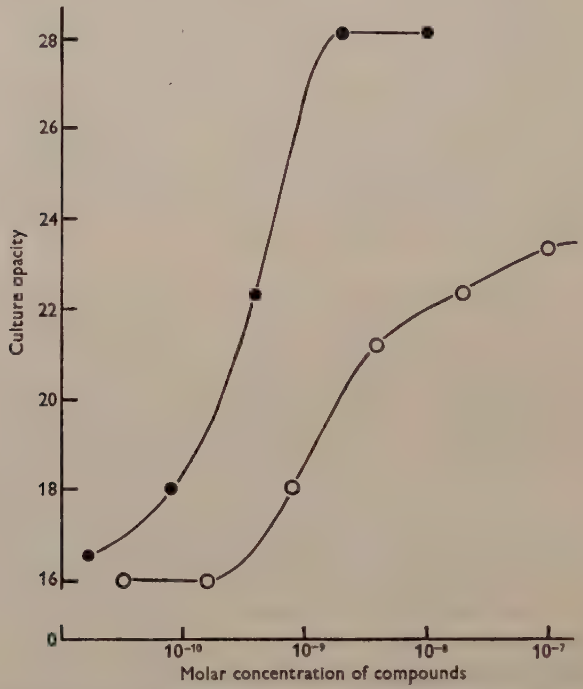


Fig. 3. **Stimulation by pteroylglutamic acid (●) and by 2-deaminopteroylglutamic acid (○) of the suboptimal growth of *L. casei* supported by a mixture of thymine and hypoxanthine.**

With *Strep. faecalis* the relationship between 2-deaminopteroylglutamic acid and pteroylglutamic acid, on the other hand, appears to be a competitive one. In the same test conditions pteroylaspartic acid (Hutchings *et al.* 1947) and several inhibitory pterins (Daniel, Norris, Scott & Heuser, 1947) showed a competitive relationship.

For the type of relationship of the analogue with rhizopterin and Leucovorin in *Strep. faecalis*, and with pteroylglutamic acid and Leucovorin in *L. casei*, no parallel can be found amongst the other reported analogues of folic acid. On the other hand, inhibition of growth of *Strep. faecalis* by 7-methylpteroylglutamic acid is overcome less efficiently by pteroylglutamic acid than by rhizopterin (Gordon, Ravel, Eakin & Shive, 1948). Similarly, natural *Leuconostoc citrovorum* factor overcomes the toxicity of 4-aminopteroylglutamic acid in the rat but pteroylglutamic acid does not (Nichol & Welch, 1950).

We can offer as yet no acceptable explanation for the stimulation of the growth of *L. casei* by 2-deaminopteroylglutamic acid in the medium which contained purine and thymine in place of folic acid. Two possibilities might be considered. The first is that *L. casei* is capable of aminating the compound and that this is a slow reaction which only proceeds at a significant rate when the population is large. It might be achieved where there is considerable growth independent of folic acid, but not where there is a small initial population and where the ratio between analogue and growth factor is an inhibitory one. The second and more remote possibility is that the thymine-purine medium contains the end-products of all but one of the reactions for which folic acid is required, and that this hypothetical reaction can be catalysed by a form of folic acid which lacks the 2-amino group. One of the difficulties of accepting this suggestion is that the organism grows in these conditions at an apparently maximal rate (although to a submaximal density) without the addition of folic acid. The last postulated function cannot therefore be regarded as essential, or else suboptimal traces of folic acid are (*a*) present in the medium, or are (*b*) synthesized by *L. casei* (cf. Rogers & Shive, 1948). All that can be said with certainty, however, is that the real explanation can only be found after further experiments.

We wish to record our gratitude to Prof. A. Albert for the interest he has shown in this work, to Dr D. D. Woods for his encouragement and many helpful suggestions, and to Mrs Doreen Wakelin for valuable technical assistance.

REFERENCES

Albert, A., Brown, D. J. & Cheeseman, G. (1952). Pteridine studies. Part III. The solubility and the stability to hydrolysis of pteridines. *J. chem. Soc.* p. 4219.

Brown, D. J. (1953). Some pteridines related to folic acid. Part I. 2-Deamino-analogues. *J. chem. Soc.* p. 1644.

Cosulich, D. B., Smith, J. M. & Broquist, H. P. (1952). Diastereoisomers of leucovorin. *J. Amer. chem. Soc.*, **74**, 4215.

Daniel, L. J., Norris, L. C., Scott, M. L. & Heuser, G. F. (1947). Growth inhibition of bacteria by synthetic pterins. Studies with *Streptococcus faecalis*, *Lactobacillus casei* and *Lactobacillus arabinosus*. *J. biol. Chem.* **169**, 689.

Gordon, M., Ravel, J. M., Eakin, R. E. & Shive, W. (1948). Formylfolic acid, a functional derivative of folic acid. *J. Amer. chem. Soc.* **70**, 878.

Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J. & SubbaRow, Y. (1947). Pteroylaspartic acid, an antagonist for pteroylglutamic acid. *J. biol. Chem.* **170**, 323.

Jukes, T. H., Franklin, A. L. & Stokstad, E. L. R. (1950). Pteroylglutamic acid antagonists. *Ann. N.Y. Acad. Sci.* **52**, 1336.

Krueger, K. & Peterson, W. H. (1945). The response of *Lactobacillus casei* and *Streptococcus faecalis* to vitamin B_6 and thymine. *J. biol. Chem.* **158**, 145.

Nichol, C. A. & Welch, A. D. (1950). On the mechanism of action of aminopterin. *Proc. Soc. exp. Biol., N.Y.* **74**, 403.

Nimmo-Smith, R. H., Lascelles, J. & Woods, D. D. (1948). The synthesis of 'folic acid' by *Streptobacterium plantarum* and its inhibition by sulphonamides. *Brit. J. exp. Path.* **29**, 264.

Oleson, J. J., Hutchings, B. L. & SubbaRow, Y. (1948). Studies on the inhibitory nature of 4-aminopteroylglutamic acid. *J. biol. Chem.* **175**, 359.

Rabinowitz, J. C. & Snell, E. E. (1947). The vitamin B_6 group. XI. An improved method for assay of vitamin B_6 with *Streptococcus faecalis*. *J. biol. Chem.* **169**, 631.

Roberts, E. C. & Snell, E. E. (1946). An improved medium for microbiological assays with *Lactobacillus casei*. *J. biol. Chem.* **163**, 499.

Rogers, L. L. & Shive, W. (1948). Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions. VII. Relationship of purines and thymine to folic acid. *J. biol. Chem.* **172**, 751.

Sauberlich, H. E. (1952). Comparative studies with the natural and synthetic citrovorum factor. *J. biol. Chem.* **195**, 337.

Stokes, J. L. (1944). Substitution of thymine for 'folic acid' in the nutrition of lactic acid bacteria. *J. Bact.* **48**, 201.

Woolley, D. W. (1952). *A study of Antimetabolites*. London: Chapman and Hall Ltd.

(*Received* 22 *June* 1953)

[*The editors of the* Journal of General Microbiology *accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from authors.*]

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Sixteenth General Meeting at the Royal Institution, London, on Monday, Tuesday and Wednesday, 13, 14 *and* 15 *April* 1953. *The following communications were made:*

COMMUNICATIONS

Effect of Aeration on the Citric Acid Metabolism of *Aerobacter aerogenes*.

By S. Dagley (*University of Leeds*) and E. A. Dawes (*University of Glasgow*)

Suspensions of *A. aerogenes*, grown without aeration on simple salt media providing citrate as sole carbon source, produced pyruvic acid rapidly on incubation with citrate, either aerobically or anaerobically. Crops grown with aeration produced pyruvate and α-ketoglutarate at a much slower rate by a process requiring oxygen. Measurements of rates of ketoacid production by cells under standard conditions of incubation with citrate showed that the enzyme responsible for dissimilation was adaptive, was developed anaerobically and was gradually lost during growth with aeration. Towards the end of the logarithmic phase of anaerobic growth in a medium containing citrate in excess, the activity of the cells decreased; this was ascribed to inhibition of citrate dissimilation by the acetic acid produced. When cells grown anaerobically in citrate medium were inoculated into growth media containing citrate and a limiting amount of glucose, the latter was utilized first and ability to dissimilate citrate was lost; activity tests showed recovery when glucose disappeared, and growth at the expense of citrate resumed after a lag period ('diauxie'). When citrate was fermented in the presence of 0·0025 M-$\mathrm{AS_2O_3}$, pyruvate accumulated and $\mathrm{CO_2}$ evolution corresponded to that calculated for dissimilation of citrate to acetate + pyruvate + $\mathrm{CO_2}$. The Pasteur effect in this system appears to depend upon two phenomena. First, two pathways for citrate breakdown exist, one favoured by aeration and the other developed in absence of air. Secondly, the anaerobic reaction is inhibited by an accumulating metabolic product, acetate, so that growth is restricted even at pH 7·0.

Some Carbohydrate-fermenting Organisms isolated from the Rumen of the Sheep.

By Sheila M. Wilson (*University of Sheffield*)

Serial dilution in shake cultures was used for the isolation of rumen organisms. Media were developed which reproduced as far as practical the conditions in the rumen, using glucose, cellobiose or grass levan as source of carbohydrate. The dilution technique gave a rough estimate of the numbers of cultivable organisms but, though several modifications of the media were tried, growth never occurred beyond the ninth 1/10 dilution. This would represent a count of the order of 10^8/ml. of rumen contents and appears to be low.

Isolations were made of four groups of organisms which seemed to form a stable part of the population of two fistulated animals during an 11-month period. Group A: these were fast growing, Gram-positive cocci which appeared to be closely related to *Streptococcus bovis*. Group B: these were small, Gram-negative rods with pointed ends which, under certain conditions of growth, appeared as large, swollen, pleomorphic forms. They were obligate anaerobes belonging to the family Pseudomonadaceae and had a single polar flagellum. They produced acetic, succinic and lactic acids from glucose. Group C: these were small, slender rods, with monotrichate flagellation, obligate anaerobes belonging to the family Pseudomonadaceae, which produced acetic, butyric and lactic acids from glucose. Group D: these were mainly cocci and short rods, but in young cultures filamentous branching rods were observed. They were Gram-negative, obligate anaerobes requiring bicarbonate for growth. Acetic, succinic and lactic acids were produced from glucose.

Three abattoir samples and single rumen samples from nine other sheep were examined and, in addition, isolations were made from the abattoir samples. The distribution of groups B–D was limited. Group A was found in all but one animal, but the numbers were relatively low, *c.* 10^5/ml. of rumen contents.

The Diaminodicarboxylic Acids of Actinomycetales. By ELIZABETH WORK (*Department of Chemical Pathology, University College Hospital Medical School, London*)

Diaminopimelic acid (DAP) $COOH.CH(NH_2).(CH_2)_3.CH(NH_2).COOH$ (Work, 1951) is confined to the Schizomycetes (excluding Gram-positive cocci and certain Actinomycetales) and to the related Myxophyceae. The technique used in examining its distribution was two-dimensional paper chromatography of the peroxide-treated neutral fraction remaining after electrodialysis of the whole cell hydrolysate. The results on the neutral fraction could be checked by the specific DAP decarboxylase extracted from *Aerobacter aerogenes*.

Among the Actinomycetales, four strains of *Mycobacterium* and four of *Nocardia* contained about 1–2 % (dry wt.) of DAP. Diaminopimelic acid was absent from three strains of *Streptomyces* and one *Actinomyces*, all of which contained low concentrations (about 0·1 %) of an unidentified amino-acid (A) with similar chromatographic properties to DAP except for a slightly higher R_F value in phenol. A was not decarboxylated by DAP decarboxylase. Purification of A was carried out by essentially the same method as was used for DAP. Analysis gave $C_4H_8NO_2$ for the empirical formula, with all N present as α-amino N (Van Slyke). The similarity in properties to DAP suggested a diaminodicarboxylic acid, the formula of which ($C_8H_{16}N_2O_4$) differed from that of DAP ($C_7H_{14}N_2O_4$) by only CH_2. Diaminosuberic acid (DAS), which is the corresponding straight chain homologue of DAP, differed from A in solubility and chromatographic behaviour, the R_F of A usually lying between those of DAP and DAS. It is therefore concluded that A must be a branched-chain homologue of DAP, possibly having a methyl side chain.

While DAP isolated from bacterial sources showed no optical rotation in any solvent, A had a rotation in 6-N HCl, $\alpha = +36{\cdot}5$.

REFERENCE

Work, E. (1951). *Biochem. J.* **49**, 17.

Acetate, Amino-acid Synthesis and the Tricarboxylic Acid Cycle in *Escherichia coli*. By K. McQuillen (*Department of Biochemistry, University of Cambridge*)

Esch. coli strain B growing exponentially in a glucose/salts medium containing C^{14}-labelled acetate, fixes C^{14} in cellular material. About 30 % of the isotope fixed from either $C^{14}H_3COONa$ or $CH_3C^{14}OONa$ is in the lipid fraction and about 60 % in protein. In non-acetate-adapted cells nine amino-acids become labelled—leucine; glutamic acid, proline, arginine; aspartic acid, threonine, isoleucine, lysine and methionine. The first four of these have specific activities which are approximately equal and several times higher than those of the remainder.

Addition to the growth medium of any of these amino-acids other than glutamic and aspartic acids, suppresses the appearance of C^{14} in only the corresponding amino-acid of the protein. Aspartic acid affects itself, threonine, isoleucine, lysine and methionine. When glutamic acid is present in the growth medium, the protein-C^{14} from $C^{14}H_3COONa$ or $CH_3C^{14}OONa$ is almost exclusively in leucine.

These and other data are consistent with the Krebs tricarboxylic acid cycle operating principally as a mechanism for amino-acid synthesis. Under the experimental conditions used, about half of the protein carbon passes through some part of the cycle while probably less than one-sixth of the glucose carbon metabolized during growth does so. About 70 % of the oxalacetate formed as an intermediate arises from CO_2 and pyruvate, 30 % from α-ketoglutarate. Glutamic acid is precursor to proline and arginine. Aspartic acid contributes four carbon atoms to threonine, isoleucine, lysine and methionine. The pathway of synthesis of leucine is unknown.

This work was carried out in collaboration with Dr R. B. Roberts of the Carnegie Institution of Washington.

Cystathionine in relation to Methionine Synthesis by *Bacterium coli*. By S. Wijesundera and D. D. Woods (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The nutritional requirements of mutants of several micro-organisms indicate that cystathionine and homocysteine are successive precursors of methionine. Cell suspensions of *Bact. coli* were found by Gibson & Woods (1952) to convert homocysteine to methionine, *p*-aminobenzoic acid and vitamin B_{12} being required; serine was the methyl donor (Gibson, 1952). The conversion of cystathionine to methionine was now studied with suspensions of several strains of this organism. Only two of the ten tested formed methionine to an extent comparable with that from homocysteine. The failure was not due to inability to form homocysteine from cystathionine since cell-free cystathionase preparations from poor methionine synthesizers were as active as those from competent strains. There is a possibility that the difference lies in the relative activity of other enzymes (e.g. desulphydrase, transaminase) competing for homocysteine formed from exogenous cystathionine.

Homocysteine, pyruvate and NH_3 are formed linearly from L-cystathionine by the cell-free enzyme. The properties of the aged and partly purified preparation (which formed no pyruvate from L-serine or DL-alanine) agree with the direct formation of these products from cystathionine; an active D-serine deaminase present was preferentially inactivated by heat. The activity of an aged, dialysed preparation was increased 200-fold by pyridoxal phosphate plus Mg ion; neither was effective alone.

Cell-free preparations of a mutant strain which grew with methionine or homocysteine, but not with cystathionine, lacked cystathionase activity when tested under conditions which showed activity with a strain which could utilize cystathionine for growth. It is being investigated further to decide whether or not it contains some altered type of enzyme.

REFERENCES

Gibson, F. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 80.
Gibson, F. & Woods, D. D. (1952). *Biochem. J.* 51, v.

Some Interrelationships between Nicotinic Acid, Pyridoxin and Purines in the Growth of a Strain of *Saccharomyces cerevisiae*. By J. W. Moulder and D. D. Woods (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Saccharomyces cerevisiae Y47 (Cutts & Rainbow, 1950) grows well aerobically at 30° and pH 6 in the presence of glucose, *p*-aminobenzoic acid, pantothenic acid, biotin, thiamine and inositol without the addition of any pyridoxin. Added adenine inhibits growth, and this inhibition is greatly increased by the simultaneous addition of nicotinic acid, which by itself has no effect. A 50% inhibition of growth is produced by 10^{-6} M adenine and nicotinic acid. The toxic effects of adenine and nicotinic acid are completely prevented by adding 10^{-9}–10^{-10} M pyridoxin to the basal medium.

Adenine and hypoxanthine are equally effective as the purine component of the inhibitory combination; other purines and pyrimidines are almost or completely inactive. Nicotinic acid may be replaced by nicotinamide, and by preparations of diphosphopyridine nucleotide and triphosphopyridine nucleotide; they are all, however, less than one-tenth as effective as nicotinic acid. Isonicotinic acid hydrazide is inactive. Pyridoxin, pyridoxal and pyridoxamine are of approximately equal effectiveness in preventing the adenine-nicotinic acid inhibition.

Growth of the *p*-aminobenzoic acid-requiring *S. cerevisiae* Y 45 is also strongly inhibited by adenine and nicotinic acid, growth of a normal laboratory strain of *S. cerevisiae* is moderately inhibited, and growth of *S. carlsbergensis* 4228 is not inhibited.

There appears to be no other comparable interrelation between adenine, nicotinic acid and pyridoxin in either animal or microbial nutrition, but Rabinowitz & Snell (1951) have reported a similar case in which the growth of *S. carlsbergensis* 4228 is inhibited by very low concentrations of thiamine and is restored by pyridoxal.

REFERENCES

Cutts, N. S. & Rainbow, C. (1950). *J. gen. Microbiol.* **4**, 150.
Rabinowitz, J. C. & Snell, E. E. (1951). *Arch. Biochem.* **33**, 472.

Characteristic Mutants of Species in the *Aspergillus niger* Group and their Behaviour in Mixed Culture. By J. L. Yuill (*John & E. Sturge Ltd., Birmingham*)

Twelve cultures from the *A. niger* group, including strains of the species *A. niger* van Tieghem, *A. awamori*, *A. foetidus*, *A. phoenicis*, *A. pulverulentus*, *A. fonsecaeus*, *A. carbonarius* and *A. japonicus*, some of which had already produced spontaneous mutants, were submitted to ultraviolet irradiation.

Several different mutants were obtained from each culture with colours ranging from near-white, pink to cinnamon; yellow, tan to light and dark brown; green, olive, and slate. In spite of great morphological and other differences between the original cultures, certain essentially similar mutants are found in a majority of them after irradiation. Thus analogues of *A. niger mut. cinnamomeus* and *A. niger mut. Schiemanni*, and a yellow-to-green type with obvious morphological modifications are common. An extreme morphological mutant, repeating the essential characters of *Cladosarium olivaceum*, appears in nine cases including the multi-nucleate species *A. carbonarius* and the species *A. japonicus* with sterigmata in one series.

The mutants were tested for acid-producing capacity alone and in mixed culture with parent strain or another mutant. Acid production in mixed culture equalled that of one or other component or was intermediate.

Dark coloured heterokaryotic conidial heads appeared on the mycelial felts of certain mixed cultures on Currie's solution. Two or more related colour mutants, grown first in mixed culture in 'shake-flasks', produce pellets of mycelium, which, on being transferred to the surface of Czapek agar and further incubated, spore readily and disclose what heterokaryons have formed. By using this technique, it was shown that many, but not all, pairs of related mutants produce heterokaryotic heads as fully pigmented as the parent, or of some lighter shade different from that of either mutant.

Nutritional Conditions which increase the Ability of *Aspergillus niger* to form α-Ketoglutarate from Citrate. By A. N. Hall and T. K. Walker (*College of Technology, Manchester*)

The formation of α-ketoglutarate from citrate by two strains of *Aspergillus niger* has been studied in arsenite-inhibited systems.

Mycelium cultivated in a glucose-containing medium in surface culture accumulated very small quantities of α-ketoglutarate from citrate in the presence of arsenite, pyruvate being the main product. Subsequently, the *A. niger* strains were trained to grow on citric acid as sole carbon-source by successive transfers through media with increasing citric acid concentrations and decreasing glucose concentrations until they developed freely in presence of citric acid monohydrate at concentration 7·5 % (w/v). Washed, citric acid-grown mycelia were allowed to float on 0·2 M sodium citrate in 0·1 M phosphate buffer containing 0·01 M potassium arsenite (pH 6·8). Following incubation at 30° for 24 hr. the metabolism solutions were filtered and the 2:4-dinitrophenylhydrazones were precipitated. In both cases a yellow product (120 mg.) was obtained from 100 ml. metabolism solution. Paper chromatography of the derivatives in *n*-butanol-ammonia-water showed them to be almost pure samples of the 2:4-dinitrophenylhydrazone of α-ketoglutarate. The derivative of pyruvate was present in traces only. After purification both samples melted at 224° alone or in admixture with an authentic sample of the 2:4-dinitrophenylhydrazone of α-ketoglutaric acid.

It is suggested that *A. niger* is able to degrade citric acid in two ways, either through acetone-dicarboxylate as demonstrated by Challenger, Klein & Walker (1927) or by way of α-ketoglutarate.

REFERENCE

Challenger, F., Klein, L. & Walker, T. K. (1927). *J. chem. Soc.* p. 3044.

A Preliminary Report of the Application of Electrokinetic Methods to the Study of Bacterial Adaptation. By A. M. James and P. J. Barry (*Chelsea Polytechnic, London*)

The electrophoretic mobilities of cells of *Aerobacter aerogenes* show a typical Gaussian distribution during growth in a wide range of natural and chemically-defined media (Barry & James, 1952, 1953). Increasing concentrations of proflavine, on resting cells, cause a linear decrease of mobility, until the cells are precipitated. This is completely reversible for on re-suspending the cells in M/150 phosphate buffer at pH 7·0 the mobility returns to the normal value for untreated cells. Adaptation of the cells to growth in concentrations in excess of 40 mg./l. produced cells with a wide distribution of mobilities, which was independent of training concentration up to 500 mg./l. Higher concentrations brought about an increased number of cells with lower mobilities. The change from the homogeneous to the heterogeneous population occurred towards the end of the lag or early in the growth phase when cells adapted to

40 mg./l. (homogeneous) were transferred to media containing 78 mg./l. proflavine. This wide distribution of mobilities was transmissible through more than seventy-five generations in drug-free medium, suggesting that the phenomenon is not due to irreversibly absorbed proflavine or to surface injury. Cells fully adapted to 155 mg./l. proflavine when plated out to give individual colonies which were then tested showed that each cell, irrespective of its own mobility, was capable of giving rise to the complete spectrum of mobilities. The proflavine acting on the bacteria has caused a modification which is then transmissible even in the absence of the drug. It is, therefore, not acting directly upon the surface but upon the deeper layers of the cell wall where the enzymes responsible for synthesizing fresh surface material are situated. 5-Aminoacridine acts in the same way and gives rise to similar phenomena.

REFERENCES

BARRY, P. J. & JAMES, A. M. (1952). *J. chem. Soc.* p. 3340.
BARRY, P. J. & JAMES, A. M. (1953). *J. chem. Soc.* p. 1264.

Apparent Adaptation to *in vivo* conditions in *Pasteurella pestis*. By T. W. BURROWS (*Microbiological Research Department, Porton*)

An investigation into the basis of virulence in *Pasteurella pestis*, using virulent and avirulent strains having apparently identical *in vitro* properties, revealed that as harvested from 18 hr. growths on tryptic meat digest agar both strains are equally susceptible to the polymorphonuclear defence mechanism of the mouse. Following intraperitoneal injection, avirulent organisms are rapidly phagocytosed over the initial 30 min. period, this high rate being maintained until the injected cells are completely eliminated. Virulent organisms similarly are rapidly phagocytosed over this initial period, after which the rate of ingestion steadily declines and ceases completely within 5 hr. Virulent cells harvested after 4–5 hr. *in vivo* are not phagocytosed at all when injected into normal mice, indicating them to have been altered in some manner which renders them resistant to phagocytosis. Avirulent cells are unable to effect such an alteration *in vivo*.

A purine requiring mutant derived from the virulent strain is unable to become phagocytosis resistant *in vivo* unless supplied with its specific growth factor, implying that active metabolism of the virulent cell is necessary for the development of resistance. In addition to becoming resistant to phagocytosis, virulent cells can inactivate polymorphs rendering them incapable of ingesting agar-grown organisms.

The Adaptive Oxidation of L-Histidine by certain Bacteria. By H. TRISTRAM (*University College, London*)

An unidentified non-sporing rod isolated from soil, when grown on a glucose-mineral salts medium containing ammonium chloride as sole source of nitrogen, exhibits a low rate of oxygen absorption when L-histidine is added to a washed suspension in phosphate buffer (pH 7·2). The rate of oxygen absorption increases until it becomes high and constant after some 6–7 hr. at 25°. During the experimental period the small increase in cell dry weight and viable numbers which occurs is insufficient to account for the increase in the rate of oxygen absorption, thus excluding the possibility of selection of a histidine-oxidizing mutant.

Cells grown in a similar medium with L-histidine as sole nitrogen source and cells shaken in phosphate buffer containing L-histidine show a high constant rate of oxygen absorption immediately after the addition of L-histidine.

Sodium azide and dinitrophenol at concentrations which have little or no effect on the rate of oxygen absorption by adapted cells completely inhibit the adaptation of unadapted cells. This evidence is in accord with the view that the phenomenon is one of induced enzyme synthesis.

There is some evidence that D-histidine may act as an enzyme inducer, although the possibility of contamination of the D isomer by the L isomer has not yet been ruled out. D-Histidine is not itself metabolized either by unadapted cells or by cells in which the enzyme has been induced by either L- or D-histidine.

The biochemical mechanism of the oxidation remains to be elucidated, but all three nitrogen atoms of the histidine molecule appear as ammonia; the oxygen absorption, the carbon dioxide output and the respiratory quotient correspond to the complete oxidation of the substrate.

Auto-adaptation: a New Phenomenon observed in a Bacterial Population.

By Barbara E. Wright (*Carlsberg Laboratory, Copenhagen*)

When a strain of *Escherichia coli* with a partial requirement for glycine is grown in liquid 'minimal' medium, the culture undergoes progressive, stepwise increases in growth rate, until glycine synthesis no longer limits growth (wild type). The stepwise increases in rate are not brought about by a simple mutation-selection mechanism, but by successive mass-transformations to cell types more efficient in glycine biosynthesis. Conversion of one cell type, A, to the succeeding, faster-growing cell type, B, occurs at a rate of 1·2 per generation of A cells. Greater capacity for glycine biosynthesis is thus produced in a discontinuous way as a result of the metabolic activity of cells already in possession of low levels of this biosynthetic ability. The working hypothesis developed suggests that a 'conversion factor' is built up (intra- or extracellularly) to a critical level at which A cells are converted to B cells. Assuming that some constitutive enzymes are formed adaptively in response to their substrates, and assuming the conversion factor to be a glycine precursor, its periodic accumulation could result in the induction of ever-increasing levels of enzymic ability for the conversion of this precursor to glycine. This interpretation can explain another characteristic of these cultures which may be analogous to more commonly described adapted cultures: they can be 'de-adapted'. De-adaptation is brought about by supplying the cells with an excess of their limiting factor, glycine, the utilization of which temporarily inhibits the growth rate and the capacity of the cells to transform in a minimal medium. Presumably glycine utilization results in the removal or inhibition of the adaptive substrate (glycine precursor) and/or its enzyme.

The Fermentation of Raffinose by *Saccharomyces fragilis* Jorgensen. By R. Davies (*Medical Research Council Unit for Chemical Microbiology, Biochemical Laboratory, University of Cambridge*)

Washed suspensions of *Saccharomyces fragilis* grown in a raffinose-Difco yeast extract-ammonia-salts medium hydrolyse raffinose to fructose and melibiose, the fructose being fermented to ethanol and carbon dioxide and the melibiose remaining unattacked. Washed suspensions of the yeast grown in a glucose medium ferment raffinose very slowly, but will ferment sucrose at the same high rate as glucose, suggesting that raffinose fermentation is an adaptive property of *S. fragilis*, whereas sucrose fermentation is a constitutive property. If sucrose and raffinose are both hydrolysed by the enzyme invertase, as is generally believed, this situation would be anomalous. However, following growth in raffinose medium the invertase content of *S. fragilis* is at least 10 times greater than that of cells grown in glucose medium and this, coupled with the slower rate of hydrolysis of raffinose (1/10) as compared with sucrose, is sufficient to explain the apparent adaptive nature of raffinose fermentation. Following growth in the yeast extract medium in the absence of added sugar, the invertase content of the cells is as high as in cells grown in raffinose medium suggesting that invertase formation in *S. fragilis* is inhibited in the presence of glucose. Washed suspensions of *S. fragilis* grown in a 10 % glucose medium for 12–24 hr. at 25° contain little invertase but synthesize it when incubated in McIlvaine buffer pH 4·5 and sucrose for 2 hr.; the ability to hydrolyse raffinose also increases in the same ratio. *S. fragilis* cells grown or treated in a variety of ways, such that the rate of sucrose hydrolysis/mg. dry wt. of cells shows a 200-fold variation, show only a twofold variation in the ratio (rate of sucrose hydrolysis)/(rate of raffinose hydrolysis). The evidence favours the view that sucrose and raffinose are hydrolysed by the same enzyme in *S. fragilis*.

Variation in a small Colony Strain of Staphylococcus. By C. H. Smith (*Institute of Ophthalmology, London*)

A small colony mutant of a coagulase positive strain of staphylococcus was obtained from the resistant organisms present after lysis of a broth culture by K phage. The organism was typical of the 'G' mutants except that its phage pattern differed from its parent and it was no longer coagulase positive or sensitive to K phage. After a period of 3 years the small colony strain, while maintaining its colonial morphology, has reverted to a form in which it is coagulase positive and sensitive to K phage and probably has a similar phage pattern to its parent strain. During this time the necessity to add uracil to fluid media in order to obtain growth has been lost.

Inhibition by Amino-acids of the Growth of Coliform Organisms. By D. Rowley (*The Wright-Fleming Institute of Microbiology, London*)

Strains of coliform organisms tested for inhibition by amino-acids on a medium in which ammonia was the sole nitrogen source, revealed that many strains were inhibited by one amino-acid or another. Each inhibition could be prevented by the simultaneous presence of one or more of the remaining amino-acids. Thus norleucine inhibition was antagonized by methionine, valine by isoleucine or leucine, serine by glycine, and cysteine by methionine.

In the case of the K 12 strain the inhibition has been shown to be bacteriostatic in nature and cultures will 'escape' from the inhibition if incubated long enough. This delayed growth is not due to the emergence of permanently resistant organisms since the final culture is as sensitive to the inhibitory effect of valine as the original. Cultures of K 12 have been assayed for valine sensitivity before, during and after escape from the inhibitory lag caused by valine. At all these times the cells had the same high sensitivity to valine as the parent K 12 strain. This suggests that it is the medium rather than the organisms which has changed by the end of the lag period.

Genetic Analysis of Valine Inhibition in the K12 strain of *Escherichia coli*. By A. Manten and D. Rowley (*National Institute of Public Health, Utrecht and The Wright-Fleming Institute of Microbiology, London*)

Small concentrations of valine are strongly inhibitory to the K 12 strain of *Escherichia coli* and also to some artificial mutants derived from K 12 (58–161 and J 5–10). One mutant, however (W 677), which requires leucine for growth is not inhibited. This may be due to the fact that leucine antagonizes the inhibitory effect of valine on K 12 or W 677 may be genotypically resistant. A cross between W 677 and 58–161 yielded only valine sensitive prototrophs, this is consistent with W 677 being merely phenotypically resistant to valine because of the necessary presence of leucine. A valine resistant mutant of the 58–161 strain was developed (58–161^R) and recombinations between this strain and the W 677 and J 5–10 mutants were made. The cross 58–161^R + J 5–10 yielded a mixture of 58 % valine sensitive prototrophs and 42 % valine resistant ones. On the other hand, 58–161^R + W 677 gave only valine resistant colonies. This can be explained by assuming a strong linkage of valine resistance to one of the factors for prototrophy. The results of crossings inoculated on to minimal medium supplemented with thiamine or threonine lead to the conclusion that valine resistance is strongly linked to the factor for leucine independence.

The Selection of Amino-acid Inhibited Mutants of *Escherichia coli*. By R. C. Clowes and D. Rowley (*The Wright-Fleming Institute of Microbiology, London*)

The inhibition of *Escherichia coli* (*Bacterium coli*) has been shown to be genetically controlled in the case of the K12 strain, in which the factor for valine resistance was shown to be closely linked with the factor for the independence of the antagonizing amino-acid, leucine. If it were possible to select from a parent stock mutants having sensitivities to other amino-acids apart from those inhibiting the parent strain, and which are still prototrophic, a new range of biochemical genetic markers would be available for further studies.

Several strains inhibited only by norleucine have been used in this investigation. Seeding an ultraviolet irradiated culture on solid medium containing an amino-acid (100 μg./ml.) should result in sensitive mutants appearing as small colonies due to inhibition by the amino-acid. Isolation and testing of small colonies developing after such treatment was first used as a preliminary method of selection. An alternative technique used a modification of Davis's penicillin method of selection for auxotrophic mutants. An irradiated culture was incubated in liquid minimal medium containing penicillin and the amino-acid. This should allow proliferation and subsequent sterilization of the parent cells by the penicillin whilst the growth of sensitive mutants should be suppressed and thus protected against penicillin action. Subsequent inoculation on nutrient agar on which inhibition is antagonized would allow growth of both parent and mutant survivors. Preliminary experiments using a mixed culture containing sensitive and uninhibited strains indicated an enrichment in the proportion of sensitive cells, although protection of these in the presence of the amino-acid was incomplete. By these methods mutants sensitive to norvaline, aspartic acid, serine and cysteine were isolated. Comparison of their characteristics with those of the parent strains confirmed their parental derivation.

SYMPOSIUM ON ADAPTATION IN MICRO-ORGANISMS

Adaptation, Evolutionary and Physiological; or Darwinism among the Micro-organisms. By R. Y. Stanier (*University of California, Berkeley*)

Observations on Bacterial Adaptation. By A. C. R. Dean and Sir Cyril Hinshelwood (*Physical Chemistry Laboratory, University of Oxford*)

The Nature of Variations affecting Bacterial Adaptability. By A. W. Ravin (*Columbia University, New York*)

Stages in Enzyme Adaptation. By M. R. Pollock (*National Institute for Medical Research, London*)

The Nature of the Precursor in the Induced Synthesis of Enzymes. By S. Spiegelman and H. O. Halvorson (*University of Illinois*)

Specific Induction and Inhibition of Enzyme Biosynthesis. By M. Cohn and J. Monod (*Pasteur Institute, Paris*)

A Specific Relation between Enzymatic Adaptation and Cytoplasmic Mutation. By P. P. SLONIMSKI (*Laboratoire de Génétique, Paris*)

The Effect of Temperature on Enzymic Adaptation, Growth and Drug Resistance. By R. KNOX (*Guy's Hospital Medical School, London*)

The Development of Drug Resistance in Micro-organisms. By E. P. ABRAHAM (*Sir William Dunn School of Pathology, Oxford*)

Antibiotic-resistant Staphylococcal Variants. By MARY BARBER (*St Thomas's Hospital Medical School, London*)

The Ecology of Tubercle Bacilli Resistant to Streptomycin and Isoniazid. By D. A. MITCHISON (*Postgraduate Medical School, London*)

Influence of Bacteriophage on Bacterial Variation and Evolution. By L. F. HEWITT (*Serum Research Institute, Carshalton*)

Adaptations in Paramecium. By G. H. BEALE (*Institute of Animal Genetics, Edinburgh*)

Environmental and other Aspects of Adaptation. By L. F. L. CLEGG (*National Institute for Research in Dairying, Shinfield*) and S. E. JACOBS (*Imperial College of Science, London*)

Ecological Adaptations in Fungi. By W. BROWN and R. K. S. WOOD (*Imperial College of Science, London*)

Index of Authors

Entries marked with an asterisk (*) are those for which only titles are given and of which there are no summaries.
Small roman numerals refer to pages in Proceedings of the Society for General Microbiology.

*Abraham, E. P. The development of drug resistance in micro-organisms. xii
Adam, K. M. *In vivo* observations on the ciliate protozoa inhabiting the large intestine of the horse 376
Anderson, E. S. & Felix, A. The Vi type-determining phages carried by *Salmonella typhi* 65
Appleby, J. *see* Heald, P. J. 207

Baker, H., Sobotka, H. & Hutner, S. H. Growth requirements of some thermophilic and mesophilic bacilli 485
*Barber, M. Antibiotic-resistant staphylococcal variants xii
Barry, P. J. *see* James, A. M. vi
*Beale, G. M. Adaptations in Paramecium xii
Bergersen, F. J. A probable growth cycle in *Bacillus megaterium* 26
Bergersen, F. J. Cytological changes induced in *Bacterium coli* by chloramphenicol 353
Brian, P. W. *see* Jefferys, E. G. 314
Briggs, M. The classification of Lactobacilli by means of physiological tests 234
Brown, D. J. *see* Nimmo-Smith, R. H. 536
*Brown, W. & Wood, R. K. S. Ecological adaptations in fungi xii
Burrows, T. W. Apparent adaptation to *in vivo* conditions in *Pasteurella pestis* vii

Cabelli, V. J. *see* Pickett, M. J. 249
Clarke, P. H. Growth of streptococci in a glucose phenolphthalein broth 350
*Clegg, L. F. L. & Jacobs, S. E. Environmental and other aspects of adaptation xii
Clowes, R. C. & Rowley, D. The selection of amino acid inhibited mutants of *Escherichia coli* x
*Cohn, M. & Monod, J. Specific induction and inhibition of enzyme biosynthesis xi
Conner, R. L., van Wagtendonk, W. J. & Miller, C. A. The isolation of a growth factor of steroid nature from lemon juice required for the growth of a strain of *Paramecium aurelia* 434
Cooper, P. D. The study of cell rupture in *Staphylococcus aureus*. 199
Cowan, S. T. Micromethod for the methyl red test 101

Dagley, S. & Dawes, E. A. Effect of aeration on the citric acid metabolism of *Aerobacter aerogenes* i
Davies, R. The fermentation of raffinose by *Saccharomyces fragilis* Jorgensen ix
Dawes, E. A. *see* Dagley, S. i
*Dean, A. C. R. & Hinshelwood, C. Observations on bacterial adaptation xi
Dewey, D. D. *see* Work, E. 394
Dewey, C. V. & Kidder, G. W. Factors affecting the requirement of *Tetrahymena pyriformis* (*geleii*) for folic acid 445
Duguid, J P. & Wilkinson, J. F. The influence of cultural conditions on polysaccharide production by *Aerobacter aerogenes* 174
Dyke, V. G. *see* Read, M. P. 1

Edwards, J. *see* Parry, W. R. 342
English, M. P. The fermentation of malt extract by an osmophilic yeast. With a note by J. T. Gunner and C. Rainbow 15

Farrant, J. L. & Rountree, P. M. Electron microscopy of a Staphylococcal bacteriophage 288
Felix, A. *see* Anderson, E. S. 65
Few, A. V. & Schulman, J. H. The absorption of Polymyxin E by bacteria and bacterial cell walls and its bactericidal action 454
Fulton, F. & Isaacs, A. Influence virus multiplication in the chick chorio-allantoic membrane 119
Fulton, F. *see* Isaacs, A. 132

Gompels, A. E. H. Antigenic relationships of swine influenza virus 140
Green, M. & Wilson, P. W. The utilization of nitrate nitrogen by the azotobacter 89
Gunner, J. T. *see* English, M. P. 15
Gyllenberg, H., Rossander, M. & Roine, P. A strain of *Lactobacillus bifidus* which requires strepogenin 190

Hall, A. N. & Walker, T. K. Nutritional conditions which increase the ability of *Aspergillus niger* to form α-ketoglutarate from citrate vi
*Halvorson, H. O. *see* Spiegelman, S. xi
Hare, R. *see* Wildy, P. 216
Heald, P. J., Krogh, N., Mann, S. O., Appleby, J. C., Masson, F. M. & Oxford A. E. A method for direct viable counts of the facultatively anaerobic microflora in the rumen of a sheep maintained on a hay diet 207
Hemming, H. G. *see* Jefferys, E. G. 314
*Hewitt, L. F. Influence of bacteriophage on bacterial variation and evolution xii
*Hinshelwood, C. *see* Dean, A. C. R. xi
Hood, A. M. *see* Lowbury, E. J. L. 524
Hutner, S. H. *see* Baker, H. 485

Isaacs, A. *see* Fulton, F. 119

Isaacs, A. & Fulton, F. Interference in the chick chorion 132

*Jacobs, S. E. *see* Clegg, L. F. L. xii

James, A. M. & Barry, P. J. A preliminary report of the application of electrokinetic methods to the study of bacterial adaptation vi

Jefferys, E. G., Brian, P. W., Hemming, H. G. & Lowe, D. Antibiotic production by the microfungi of acid heath soils 314

Johnstone, K. I. Micromanipulation on an agar surface for the isolation and cultivation of single organisms 293

Kassanis, B. Some effects of sucrose and phosphorus in increasing the multiplication of tobacco mosaic virus in detached tobacco leaves 467

Kidder, G. W. *see* Dewey, V. C. 445

*Knox, R. The effect of temperature on enzymic adaptation, growth and drug resistance xii

Krogh, N. *see* Heald, P. J. 207

Lederberg, J. *see* Stocker, B. A. D. 410

Levy, E., Rippon, J. & Williams, R. E. O. The relation of bacteriophage pattern to some biological properties of staphylococci 97

Lewin, J. C. Heterotrophy in diatoms 305

Lindegren, C. C. & Pittman, D. D. The induction in a *Saccharomyces* sp. of the gene-mutation controlling utilization of galactose by exposure to galactose 494

Lowbury, E. J. L. & Hood, A. M. The acquired resistance of *Staphylococcus aureus* to bacteriophage 524

Lowe, D. *see* Jefferys, E. G. 314

Lumb, M. *see* Rolinson, G. N. 385

McLean, S. J. The identification of strains of *Streptococcus pyogenes* 5, 11, 12, 27 and 44 by the precipitin test for the T. antigen 110

McQuillen, K. Acetate, amino acid synthesis and the bicarboxylic acid cycle in *Escherichia coli* iii

Mann, S. O. *see* Heald, P. J. 207

Manten, A. & Rowley, D. Genetic analysis of valine inhibition in the K12 strain of *Bacterium coli* 226

Manten, A. & Rowley, D. Genetic analysis of valine inhibition in the K12 strain of *Escherichia coli* x

Masson, F. M. *see* Heald, P. J. 207

Miller, C. A. *see* Conner, R. L. 434

Mitchell, P. Transport of phosphate across the surface of *Micrococcus pyogenes*: nature of the cell 'inorganic phosphate' 273

Mitchell, P. & Moyle, J. M. Paths of phosphate transfer in *Micrococcus pyogenes* phosphate turnover in nucleic acids and other fractions 257

*Mitchison, D. A. The ecology of tubercle bacilli resistant to streptomycin and isoniazid xii

Mitson, G. W. *see* Rainbow, C. 371

Monod, J. *see* Cohn, M. xi

Moulder, J. W. & Woods, D. D. Some interrelationships between nicotinic acid, pyridoxin and purines in the growth of a strain of *Saccharomyces cerevisiae* iv

Moyle, J. M. *see* Mitchell, P. 257

Newcombe, H. B. Radiation-induced instabilities in streptomyces 30

Newton, B. A. The release of soluble constituents from washed cells of *Pseudomonas aeruginosa* by the action of Polymyxin 54

Nimmo-Smith, R. H. & Brown, D. J. Some effects of 2-deaminopteroylglutamic acid upon bacterial growth 536

Oxford, A. E. *see* Heald, P. J. 207

Parry, W. R. & Edwards, J. The induction of lysogenesis in *Salmonella typhimurium* 342

Pickett, M. J. & Cabelli, V. J. The precipitating antigens of Friedlander's bacillus 249

Pittman, D. D. *see* Lindegren, C. C. 494

*Pollock, M. R. Stages in enzyme adaptation xi

Postgate, J. R. On the nutrition of *Desulphovibrio desulphuricans*: a correction 440

Rainbow, C. *see* English, M. P. 15

Rainbow, C. & Mitson, G. W. Nutritional requirements of acetic acid bacteria 371

*Ravin, A. W. The nature of variations affecting bacterial adaptability xi

Read, M. P. The establishment of serologically identifiable strains of *Rhizobium trifoli* in field soils in competition with the native microflora. With a note by G. V. Dyke 1

Record, B. R. & Taylor, R. Some factors influencing the survival of *Bacterium coli* on freeze drying 475

Rippon, J. E. *see* Levy, E. 97

Roine, P. *see* Gyllenberg, H. 190

Rolinson, G. N. & Lumb, M. The oxidation of lard oil by *Penicillium chrysogenum* 385

Rossander, M. *see* Gyllenberg, H. 190

Rountree, P. M. *see* Farrant, J. L. 288

Rowley, D. Interrelationships between amino acids in the growth of coliform organisms 37

Rowley, D. Inhibition by amino acids of the growth of coliform organisms x

Rowley, D. *see* Clowes, R. C. x

Rowley, D. *see* Manten, A. 226

Rowley, D. *see* Manten, A. x

Salton, M. R. J. Cell structure and the enzymic lysis of bacteria 512

Schulman, J. H. *see* Few, A. V. 454

*Slonimski, P. P. A specific relation between enzymic adaptation and cytoplasmic mutation xii

Smith, C. H. Variation in a small colony strain of staphylococcus ix

Sobotka, H. *see* Baker, H. 485

*Spiegelman, S. & Halvorson, H. A. The nature of the precursor in the induced synthesis of enzymes xi

*Stanier, R. Y. Adaptation, evolutionary and physiological; or Darwinism among the micro-organisms xi

Stocker, B. A. D., Zinder, N. D. & Lederberg, J. Transduction of flagellar characters in Salmonella 410

Sugden, B. The cultivation and metabolism of oligotrich protozoa from the sheep's rumen 44

Taylor, R. *see* Record, B. R. 475

Tristram, H. The adaptive oxidation of L-histidine by certain bacteria vii

Vincent, J. M. & Waters, L. M. The influence of the host on competition amongst clover root-nodule bacteria 357

Wagtendonk, W. J. van *see* Conner, R. L. 434

Walker, T. K. *see* Hall, A. N. vi

Waters, L. M. *see* Vincent, J. M. 357

Wells, A. Q. *Mycobacterium tuberculosis* var. *muris* 149

Wijesundera, S. & Woods, D. D. Cystathionine in relation to methionine synthesis by *Bacterium coli* iii

Wildy, P. & Hare, R. The effect of fatty acids on the growth, metabolism and morphology of the anaerobic cocci 216

Wilkinson, J. F. *see* Duguid, J. P. 174

Williams, R. E. O. *see* Levy, E. 97

Wilson, P. W. *see* Green, M. 89

Wilson, S. M. Some carbohydrate-fermenting organisms isolated from the rumen of the sheep i

*Wood, R. K. S. *see* Brown, W. xiv

Woods, D. D. The integration of research on the nutrition and metabolism of micro-organisms 151

Woods, D. D. *see* Wijesundera, S. iii

Woods, D. D. *see* Moulder, J. W. iv

Work, E. The diaminodicarboxylic acids of actinomycetales ii

Work, E. & Dewey, D. L. The distribution of α,ϵ-diaminopimelic acid among various micro-organisms 394

Wright, B. E. Auto-adaptation a new phenomenon observed in a bacterial population viii

Yuill, J. L. Characteristic mutants of species in the *Aspergillus niger* group and their behaviour in mixed culture v

Zinder, N. D. *see* Stocker, B. A. D. 410

Index of Subjects

Entries marked with an asterisk (*) are those for which only titles are given and of which there are no summaries.

Small roman numerals refer to pages in Proceedings of the Society for General Microbiology

Absidia spp. In acid heath soils, antibiotic production by (Jefferys *et al.*) 314
Absorption of Polymyxin E by bacteria and bacterial cell walls, and bactericidal action (Few & Schulman) 454
Acetic acid bacteria, nutritional requirements of (Rainbow & Mitson) 371
Acetobacter spp. nutritional requirements of (Rainbow & Mitson) 371
Acids, fatty, effect on growth, metabolism and morphology of anaerobic cocci. (Wildy & Hare) 216
Actinomycetales, diaminocarboxylic acid of (Work) ii
Adaptation, apparent, of *Past. pestis* to *in vivo* conditions, (Burrows) vii
Adaptation, auto-; new phenomenon in a bacterial population (Wright) vii
Adaptation, bacterial, electrokinetic method in study of (James & Barry) vi
*Adaptation, bacterial, observations on (Dean & Hinshelwood) xi
*Adaptation, environmental aspects of (Clegg) xii
*Adaptation, enzymatic, and cytoplasmic mutation, specific relation between (Slonimski) xii
*Adaptation, enzymic, growth and drug resistance, effect of temperature on (Knox) xii
*Adaptation, enzyme (Pollock) xi
*Adaptation, evolutionary and physiological in micro-organisms (Stanier) xi
Adaptive oxidation of L-histidine by certain bacteria (Tristram) vii
Aerobacter aerogenes, effect of aeration on citric acid metabolism of (Dagley & Dawes) i
Aerobacter aerogenes, influence of cultural conditions on polysaccharide production by (Duguid & Wilkinson) 174
Amino acid inhibited mutants of *Esch. coli*, selection of (Clowes & Rowley) x
Amino acids, interrelationships in growth of coliform organisms (Rowley) 37
Amino acid synthesis, acetate and tricarboxylic acid cycle in *Esch. coli* (McQuillen) iii
Amino acids, growth inhibition of coliform organisms by (Rowley) x
Anaerobic cocci, effect of fatty acids on growth, metabolism and morphology of (Wildy & Hare) 216
Anaerobic rumen microflora, a method for direct viable counts of the facultative (Heald *et al.*) 207
Antigens, precipitating, of Friedlander's bacillus (Pickett & Cabelli) 249
Aspergillus niger, effect of nutrient conditions on formation of L-ketoglutarate from citrate (Hall & Walker) vi
Auto-adaptation; new phenomenon in a bacterial population (Wright) viii
Azotobacter sp., utilization of nitrate nitrogen by (Green & Wilson) 89

Bacillus megaterium, cell structure and enzymic lysis of (Salton) 512
Bacillus megaterium, growth cycle in (Bergersen) 26
Bacillus spp., growth requirements of thermophilic and mesophilic (Baker, Sobotka & Hutner) 485
Bacillus subtilis, absorption of polymyxin E by (Few & Schulman) 454
Bacterial adaptation, electrokinetic methods in study of (James & Barry) vi
Bacteriophage, acquired resistance of *Staphylococcus aureus* to (Lowbury & Hood) 524
Bacteriophage, influence on bacterial variation and evolution (Hewitt) xii
Bacteriophage pattern, relation to some biological properties of staphylococci (Levy, Rippon & Williams) 97
Bacteriophage, staphylococcal, electron microscopy of (Farrant & Rountree) 288
Bacterium coli, amino acid inter-relationships in growth of (Rowley) 37
Bacterium coli, cell structure and enzymic lysis of (Salton) 512
Bacterium coli, cytological changes induced in, by chloramphenicol (Bergersen) 353
Bacterium coli, factors influencing survival (Record & Taylor) 475
Bacterium coli, genetic analysis of valine inhibition in K12 strain (Manten & Rowley) 226
Bacterium coli, see also Escherichia coli

Carbohydrate-fermenting organisms from sheep rumen (Wilson) i
Cell rupture in *Staphylococcus aureus*, the study of (Cooper) 199
Cell structure and enzymic lysis of bacteria (Salton) 512
Cell walls and enzymic lysis of bacteria (Salton) 512
Chick chorion, interference in (Isaacs & Fulton) 132
Chloramphenicol, cytological changes induced in *Bacterium coli*, by (Bergersen) 353
Chorio-allantoic membrane, of chick, apparatus for cultivating influenza virus (Fulton & Isaacs) 119
Chorio-allantoic membrane, chick, influenza virus multiplication in (Fulton & Isaacs) 119; (Isaacs & Fulton) 132
Chorion, chick, interference in (Isaacs & Fulton) 132

Ciliate protozoa inhabiting large intestine of horse (Adam) 376
Citrate, formation of L-ketoglutarate from, by *Asp. niger* (Hall & Walker) vi
Citric acid metabolism of *Aerobacter aerogenes*, effect of aeration (Dagley) i
Classification of lactobacilli by physiological tests (Briggs) 234
Clover root-nodule bacteria, influence of the host on competition amongst (Vincent & Waters) 357
Cocci, anaerobic, effect of fatty acids on growth metabolism and morphology of (Wildy & Hare) 216
Coliform organisms, amino-acids inter-relationship (Rowley) 37
Coliform organisms, growth inhibition by amino-acids (Rowley) x
Competition amongst clover-root nodule bacteria, influence of host on (Vincent & Waters) 357
Coriothyrium sp. in acid heath soils, antibiotic production by (Jefferys *et al.*) 314
Cycloposthium spp. inhabiting the large intestine of horse (Adam) 376
Cystathionine in relation to methionine synthesis in *Esch. coli* (Wijesundera & Woods) iii
Cytological changes induced in *Bacterium coli* by chloramphenicol (Bergersen) 353
Cytology and enzymic lysis of bacteria (Salton) 512
*Cytoplasmic mutation and enzymatic adaptation, specific relation between (Slonimski) xii

*Darwinism among micro-organisms (Stanier) xi
2-Deaminopteroylglutamic acid, effects on bacterial growth (Nimmo-Smith & Brown) 536
Diaminocarboxylic acids of Actinomycetales (Work) ii
Diaminopimelic acid in some intestinal micro-organisms from farm animals (Synge) 407
α,ϵ-diaminopimelic acid distribution among various micro-organisms (Work & Dewey) 394
α,ϵ-diaminopimelic acid in micro-organisms, method for identification of (Work & Dewey) 394
Diatoms, heterotrophy in (Lewin) 305
*Drug resistance in micro-organisms, development of (Abraham) xii

Electrokinetic methods in study of bacterial adaptation (James & Berry) vi
Electron microscopy of a staphylococcal bacteriophage (Farrant & Rountree) 288
Entodinium spp. from sheep rumen, cultivation and metabolism of (Sugden) 44
Enzyme adaptation (Pollock) xi
*Enzyme biosynthesis, specific induction and inhibition of (Cohn & Monod) xi
*Enzyme synthesis, induced, nature of precursor in (Spiegelman & Halvorson) xi
Escherichia coli, acetate, amino-acid synthesis and tricarboxylic acid cycle in (McQuillen) iii
Escherichia coli, K12 genetic analysis of valine inhibition (Manten & Rowley) x
Escherichia coli, selection of amino acid inhibited mutants (Clowes & Rowley) x

Fatty acids, effect on growth, metabolism and morphology of anaerobic cocci (Wildy & Hare) 216
Fermentation of malt extract by an osmophilic yeast (English) 15
Fermentation of raffinose by *Sacch. fragilis* (Davies) ix
Flagella characters in Salmonella, transduction of (Stocker, Zinder & Lederberg) 410
Folic acid, factors affecting the requirements of *Tetrahymena pyriformis* (*geleii*) for (Dewey & Kidder) 445
Folic acid, pteroic acid and pteroylglutamic acid, effects of bacterial growth (Nimmo-Smith & Brown) 536
Freeze-drying, factors influencing survival of *Bacterium coli* on (Record & Taylor) 475
Friedlander's bacillus, precipitating antigens of (Pickett & Cabelli) 249
*Fungi, ecological adaptation in (Brown & Wood) xii
Fungi of acid heath soils, antibiotic production by (Jefferys *et al.*) 314

Galactose utilization in a *Saccharomyces* sp., induction of mutation to (Lindegren & Pittman) 494
Genetic analysis of valine inhibition in *Bacterium coli* strain K12 (Manten & Rowley) 226
Genetic analysis of valine inhibition in *Esch. coli* K12 (Manten & Rowley) x
Genetics of flagella characters in Salmonella (Stocker, Zinder & Lederberg) 410
Gliocladium sp. in acid heath soils, antibiotic production by (Jefferys *et al.*) 314
Glucose phenolphthalein broth, growth of streptococci in (Clarke) 350
Growth, bacterial effects of 2-deaminopteroylglutamic acid on (Nimmo-Smith & Brown) 536
Growth cycle in *Bacillus megaterium* (Bergersen) 26
Growth factor, steroid, required for growth of *Paramecium aurelia*, strain, isolation from lemon juice (Conner, Wagtendonk & Miller) 434
Growth factors, metabolic function of (Woods) 151
Growth of anaerobic cocci, effect of fatty acids on (Wildy & Hare) 216
Growth of coliform organisms, amino acid inter-relationships (Rowley) 37
Growth requirements of thermophilic and mesophilic bacilli (Baker, Sobotka & Hutner) 485

Hay diet; method for direct viable counts of the facultatively anaerobic rumen microflora of sheep maintained on a (Heald *et al.*) 207

Heath soils, antibiotic production by the microfungi of acid (Jefferys *et al.*) 314
Heterotrophy in diatoms (Lewin) 305
Horse, observations on the ciliate protozoa inhabiting the large intestine of (Adam) 376
Humidity, effect on fermentation of malt extract by an osmophilic yeast (English) 15

*Induced synthesis of enzymes, nature of precursor in (Spiegelman & Halvorson) xi
*Induction and inhibition, specific, of enzyme synthesis (Cohn & Monod) xi
Induction of lysogenesis in *Salmonella typhimurium* (Parry & Edwards) 342
Induction of mutation to galactose utilization in a *Saccharomyces* sp. (Lindegren & Pittman) 494
Influenza virus, antigenic relationships of swine and human (Gompels) 140
Influenza virus, multiplication in chick chorio-allantoic membrane (Fulton & Isaacs) 119; (Isaacs & Fulton) 132
Inhibition of growth of coliform organisms by amino acids (Rowley) x
Instabilities in Streptomyces, radiation-induced (Newcombe) 30
Intestinal micro-organisms from farm animals, occurrence of diaminopimelic acid in (Synge) 407
Intestine of horse, observations on the ciliate protozoa inhabiting (Adam) 376
Isolation and cultivation of single organisms by micro-manipulation on agar surfaces (Johnstone) 293

K12 strain, *Bacterium coli*, genetic analysis of valine inhibition in the (Manten & Rowley) 226

Lactobacilli, classification by physiological tests (Briggs) 234
Lactobacillus bifidus strain which requires strepogenin (Gyllenberg, Rossander & Roine) 190
Lactobacillus casei, effects of pteroic and pteroylglutamic acid on growth of (Nimmo-Smith & Brown) 536
Lard oil, oxidation by *Penicillium chrysogenum* (Rolinson & Lumb) 385
Lemon juice, isolation of steroid growth factor for *Paramecium aurelia* from, (Conner, Wagtendonk & Miller) 434
Leucine independence of *Bacterium coli* K12, genetic analysis of (Manten & Rowley) 226
L-histidine, adaptive oxidation by bacteria (Tristram) vii
L-ketoglutarate from citrate, effect of nutrients on formation by *Asp. niger* (Hall & Walker) vi
Lysis, enzymic, of bacteria and cell structure (Salton) 512
Lysogenesis in *Salmonella typhimurium*, induction of (Parry & Edwards) 342
Lysozyme, effect on cell structure (Salton) 512

Malt extract, fermentation by an osmophilic yeast (English) 15
Marjory Stephenson Memorial Lecture (Woods) 151
Mesophilic and thermophilic bacilli, growth requirements of (Baker, Sobotka & Hutner) 485
Metabolism and nutrition of micro-organism, the integration of research on (Woods) 151
Metabolism of anaerobic cocci, effect of fatty acids on (Wildy & Hare) 216
Metadinium medium from sheep rumen, cultivation and metabolism of (Sugden) 44
Methods, electrokinetic, in study of bacterial adaptation (James & Barry) vi
Methyl red test, micromethod for (Cowan) 101
Micrococcus lysodeikticus, cell structure and enzymic lysis of (Salton) 512
Micrococcus pyogenes, transport of phosphate through the surface of (Mitchell) 273
Micrococcus pyogenes, paths of phosphate turnover in nucleic acids and other fractions in (Mitchell & Moyle) 257
Microfungi of acid heath soils, antibiotic production by (Jefferys *et al.*) 314
Micromanipulation on agar surfaces for isolation and cultivation of single organisms (Johnstone) 293
Micromethod for methyl red test (Cowan) 101
Morphological changes induced in *Bacterium coli* by chloramphenicol (Bergersen) 353
Morphology of anaerobic cocci, effect of fatty acids on (Wildy & Hare) 216
Mortierella alpina in acid heath soils, antibiotic production by (Jefferys *et al.*) 314
Mucor rammannianus in acid heath soils, antibiotic production by (Jefferys *et al.*) 314
Mutants of *Aspergillus niger* group, behaviour in mixed culture (Yuill) v
Mutants of *Esch. coli*, selection of amino acid inhibited (Clowes & Rowley) x
Mutation to galactose utilization in a *Saccharomyces* sp., induction of (Lindegren & Pittman) 494
Mycobacterium tuberculosis var. *muris*. (Wells) 149

Navicula spp., heterotrophy in (Lewin) 305
Nicotinic acid, pyridoxin and purines in growth of *Sach. cervisiae* (Moulder & Woods) iv
Nitrate nitrogen, utilization by *Azotobacter* sp. (Green & Wilson) 89
Nitrogen of nitrate, used by *Azotobacter* sp. (Green & Wilson) 89
Nitzschia spp. heterotrophy in (Lewin) 305
Nucleic acids and other fractions, paths of phosphate transfer in *Micrococcus pyogenes* in (Mitchell & Moyle) 257
Nutrition of *Desulphovibrio desulphuricans*: a correction on the nutrition of (Postgate) 440
Nutrition and metabolism of micro-organisms, integration of research on (Woods) 151
Nutritional requirements of acetic acid bacteria (Rainbow & Mitson) 371

Oleic acid, oxidation by *Penicillium chrysogenum* (Rolinson & Lumb) 385
Oligotrich protozoa from sheep rumen, cultivation and metabolism of (Sugden) 44
Osmophilic yeast, fermentation of malt extract by (English) 15
Osmotic barrier in *Micrococcus pyogenes* and phosphate transport (Mitchell) 273
Oxidation, adaptive of L-histidine by certain bacteria (Tristram) vii
Oxidation of lard oil by *Penicillium chrysogenum* (Rolinson & Lumb) 385

*Paramecium, adaptation in (Beale) xii
Paramecium aurelia, isolation from lemon juice of steroid growth factor for (Conner, Wagtendonk, & Miller) 434
Pasteurella pestis, apparent adaptation to *in vitro* conditions (Burrows) vii
Penicillium chrysogenum, oxidation of lard oil by (Rolinson & Lumb) 385
Penicillium spp. in acid heath soils, antibiotic production by (Jefferys *et al.*) 314
Phages carried by *Salmonella typhi*, the Vitype-determining (Anderson & Felix) 65
Phenolphthalein broth, growth of streptococci in a glucose (Clarke) 350
Phosphate transfer in *Micrococcus pyogenes*: paths of phosphate turnover in nucleic acids and other fractions (Mitchell & Moyle) 257
Phosphate transport through surface of *Micrococcus pyogenes* (Mitchell) 273
Phosphorus, effect on multiplication of tobacco mosaic virus in detached leaves (Kassanis) 467
Podsol soils, antibiotic production by microfungi of (Jefferys *et al.*) 314
Polymyxin E, absorption by bacteria and bacterial cell walls, and bactericidal action of (Few & Schulman) 454
Polymyxin, release of constituents from cells of *Pseudomonas seruginosa* by action of (Newton) 54
Polysaccharide production by *Aerobacter aerogenes*, influence of cultural conditions on (Duguid & Wilkinson) 174
Polysaccharide production by *Aerobacter aerogenes*, methods for chemical estimation of (Duguid & Wilkinson) 174
Precipitin test for T antigen, identification of *Streptococcus pyogenes* by (McLean) 110
*Precursor induced synthesis of enzymes, nature of (Spiegelman & Halvorson) xi
Protozoa, ciliate, inhabiting the large intestine of horse (Adam) 376
Protozoa, oligotrich, from sheep rumen, cultivation and metabolism of (Sugden) 44
Pseudomonas aeruginosa, release of cell constituents, by polymyxin (Newton) 54
Pseudonomas denitrificans, absorption of polymyxin E by (Few & Schulman) 454
Pseudomonas fluorescens, cell structure and enzymic lysis of (Salton) 512
Pteroic acid, effect on bacterial growth (Nimmo-Smith & Brown) 536
Pteroylglutamic acid, effect of bacterial growth (Nimmo-Smith & Brown) 536
Purines, pyridoxin and nicotinic acid, in growth of *Sacch. cerevisaie* (Moulder & Woods) iv
Pyridoxin, nicotinic acid and purines, in growth of *Sacch. cerevisiae* (Moulder & Woods) iv
Pyruvate oxidation, factor in metabolism (Woods) 151

Radiation-induced instabilities in Streptomyces (Newcombe) 30
Raffinose, fermentation by *Sacch. fragilis* (Davies) ix
Resistance, acquired, of *Staphylococcus aureus* to bacteriophage (Lowbury & Hood) 524
Resistance to valine by *Bacterium coli* K12, genetic analysis of (Manten & Rowley) 226
Rhizobium trifolii establishment in soils; note on statistical examination of establishment percentages (Dyke) 12
Rhizobium trifolii, establishment of serologically identifiable strains in field soils (Read) 1
Rhizobium trifolii, influence of the host on competition amongst strains of (Vincent & Waters) 357
Rhizobium trifolii, method for preparing specific antisera (Read) 1
Root-nodule bacteria of clover, influence of host on competition amongst (Vincent & Waters) 357
Rumen microflora, method for direct viable counts of facultatively anaerobic (Heald *et al.*) 207
Rumen of sheep, carbohydrate-fermenting organisms from (Wilson) i
Rumen, sheep, cultivation and metabolism of oligotrich protozoa from (Sugden) 44
Rupture of cells, of *Staphylococcus aureus*, study of (Cooper) 199

Saccharomyces cerevisiae, interactions between nicotinic acid, pyridoxin and purines in growth of (Moulder & Woods) iv
Saccharomyces fragilis, raffinose fermentation by (Davies) ix
Saccharomyces rouxii, fermentation of malt extract by (English) 15
Saccharomyces rouxii, vital staining of (Gunner & Rainbow) 25
Saccharomyces sp. induction of mutation to galactose utilization in (Lindegren & Pittman) 494
Salmonella, transduction of flagella characters in (Stocker, Zinder & Lederberg) 410
Salmonella typhi, Vi type-determining phages carried by (Anderson & Felix) 65
Salmonella typhimurium, induction of lysogenesis in (Parry & Edwards) 342
Salmonella typhimurium, transduction of flagella characters in (Stocker, Zinder & Lederberg) 410
Sheep rumen, carbohydrate-fermenting organisms from (Wilson) i

Sheep rumen, cultivation and metabolism of oligotrich protozoa from (Sugden) 44

Sheep rumen, facultatively anaerobic microflora, method for direct viable counts of (Heald *et al.*) 207

Shikimic acid, role in aromatic biosynthesis (Woods) 151

Single organisms, micromanipulation on agar surfaces for isolation and cultivation (Johnstone) 293

Sitosterol, growth factor for growth of *Paramecium aurelia* (Conner, Wagtendonk & Miller) 434

Small colony strain of staphylococcus, variation in (Smith) ix

Soils, acid heath, antibiotic production by microfungi of (Jefferys *et al.*) 314

Soils, establishment of serologically identifiable strains of *Rhizobium trifolii* in (Read) 1

*Staphylococcal antibiotic-resistant (Barber) xii

Staphylococcal bacteriophage, electron microscopy of (Farrant & Rountree) 288

Staphylococci, relation of bacteriophage pattern to some biological properties of (Levy, Rippon & Williams) 97

Staphylococcus aureus, acquired resistance to bacteriophage (Lowbury & Hood) 524

Staphylococcus aureus, cell structure and enzymic lysis of (Salton) 512

Staphylococcus aureus, study of cell rupture in (Cooper) 199

Staphylococcus, see also Micrococcus

Staphylococcus, variation in small colony strain of (Smith) ix

Stephenson, Marjory, Memorial Lecture, the inaugural (Woods) 151

Steroid growth factor for growth *Paramecium aurelia*, isolation from lemon juice (Conner, Wagtendonk & Miller) 434

Streptococci in glucose phenolphthalein broth, growth of (Clarke) 350

Streptococcus faecalis, absorption of polymyxin E by (Few & Schulman) 454

Streptococcus faecalis R, effects of pteroic and pteroylglutamic acid on growth of (Nimmo-Smith & Brown) 536

Streptococcus pyogenes, identification of types by precipitin test for T antigen (McLean) 110

Streptomyces radiation-induced instabilities in (Newcombe) 30

Streptomycin, effect on oligotrich protozoa from sheep rumen (Sugden) 44

Sucrose, effects on multiplication of tobacco mosaic virus in detached leaves (Kassanis) 467

Survival of *Bacterium coli* on freeze-drying, factors influencing (Record & Taylor) 475

Swine influenza virus, antigenic relationships of (Gompels) 140

T antigen, identification of *Streptococcus pyogenes* types by precipitin test for (McLean) 110

Tetrahymena pyriformis (*geleii*) factors affecting the requirements for folic acid (Dewey & Kidder) 445

Thermophilic bacilli, growth requirements of mesophilic and (Baker, Sobotka & Hutner) 485

Tobacco mosaic virus in detached leaves, effects of sucrose and phosphorus in multiplication of (Kassanis) 467

Transduction of flagella characters in Salmonella (Stocker, Zinder & Lederberg) 410

Tricarboxylic acid cycle, acetate and amino acid synthesis in *Esch. coli* (McQuillen) iii

Trichoderma viride, in acid heath soils antibiotic production by (Jefferys *et al.*) 314

Trypan Blue for vital staining of *Saccharomyces rouxii* (Gunner & Rainbow) 25

Trypsin, effect on cell structure (Salton) 512

*Tubercle bacilli resistant to streptomycin and isoniazid, ecology of (Mitchison) xii

Valine inhibition in *Esch. coli* K12, genetic analysis of (Manten & Rowley) x

Valine inhibition of *Bacterium coli* K12, genetic analysis of (Manten & Rowley) 226

Variation in small colony strain of staphylococcus (Smith) ix

*Variations affecting bacterial adaptability, nature of (Ravin) xi

Variations induced in Streptomyces by radiations (Newcombe) 30

Viable counts of facultatively anaerobic rumen microflora, a method for direct (Heald *et al.*) 207

Virus, influenza, multiplication in chick chorio-allantoic membrane (Fulton & Isaacs) 119, (Isaacs & Foulton) 132

Virus, swine and human influenza, antigenic relationships (Gompels) 140

Virus, tobacco mosaic, effects of sucrose and phosphorus on multiplication (Kassanis) 467

Vital staining for *Saccharomyces rouxii*, note on method (Gunner & Rainbow) 25

Vi type-determining phages carried by *Salmonella typhi* (Anderson & Felix) 65

Voles, *Mycobacterium tuberculosis* var *muris* in (Wells) 149

Yeast, osmophilic, fermentation of malt extract by (English) 15

Zygorhyncus moelleri in acid heath soils, antibiotic production by (Jefferys *et al.*) 314